

GRF ANALOGS WITH INCREASED BIOLOGICAL POTENCY

BACKGROUND OF THE INVENTION

(a) Field of the Invention

5 The invention relates to hydrophobic GRF analogs with increased biological potency and prolonged activity, their application as anabolic agents and treatment of growth hormone deficiencies.

(b) Description of Prior Art

10 Growth hormone (GH) or somatotropin, secreted by the pituitary gland constitutes a family of hormones which biological activity is fundamental for the linear growth of a young organism but also for the maintenance of the integrity at its adult state. GH acts directly or indirectly on the peripheral organs by stimulating the synthesis of growth factors (insulin-like growth factor-I or IGF-I) or of their receptors (epidermal
15 growth factor or EGF). The direct action of GH is of the type referred to as anti-insulinic, which favors the lipolysis at the level of adipose tissues. Through its action on IGF-I (somatomedin C) synthesis and secretion, GH stimulate the growth of the cartilage and the bones (structural growth), the protein synthesis and the cellular proliferation in multiple peripheral organs,
20 including muscles and the skin. Through its biological activity, GH participates within adults in the maintenance of a protein anabolism state, and plays a primary role in the tissue regeneration phenomenon after a trauma.

25 The decrease of GH secretion with age, demonstrated in humans and animals, favors a metabolic shift towards catabolism which initiates or participates in the aging of an organism. The loss in muscle mass, the accumulation of adipose tissue, the bone demineralization, the loss of tissue regeneration capacity after an injury, which are observed in elderly, correlate with the decrease in the secretion of GH.

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GH is thus a physiological anabolic agent absolutely necessary for the linear growth of children and which controls the protein metabolism in adults.

The secretion of GH by the pituitary gland is principally
5 controlled by two hypothalamic peptides, somatostatin and growth hormone releasing factor (GRF). Somatostatin inhibits its secretion, whereas GRF stimulates it.

The human GH has been produced by genetic engineering for about ten years. Until recently most of the uses of GH were concerned with
10 growth delay in children and now the uses of GH in adults are being studied. The pharmacological uses of GH and GRF may be classified in the following three major categories.

Children growth

Treatments with recombinant human growth hormone have been
15 shown to stimulate growth in children with pituitary dwarfism, renal insufficiencies, Turner's syndrome and short stature. Recombinant human GH is presently commercialized as an "orphan drug" in Europe and in the United States for children's growth retardation caused by a GH deficiency and for children's renal insufficiencies. The other uses are under clinical
20 trial investigation.

Long term treatment for adults and elderly patients

A decrease in GH secretion causes changes in body composition during aging. Preliminary studies of one-year treatment with recombinant human GH reported an increase in the muscle mass and in the thickness of
25 skin, a decrease in fat mass with a slight increase in bone density in a population of aged patients. With respect to osteoporosis, recent studies suggest that recombinant human GH does not increase bone mineralization but it is suggested that it may prevent bone demineralization in post-

menopausal women. Further studies are currently underway to demonstrate this theory.

Short term treatment in adults and elderly patients

In preclinical and clinical studies, growth hormone has been
5 shown to stimulate protein anabolism in wound and bone healing in cases of burn, AIDS and cancer.

GH and GRF are also intended for veterinary pharmacological uses. Both GH and GRF stimulate growth in pigs during its fattening period by favoring the deposition of muscle tissue instead of adipose tissue and
10 increase milk production in cows, and this without any undesired side effects which would endanger the health of the animals, and without any residue in the meat or milk being produced. The bovine somatotropin (BST) is presently commercialized in the United States.

Most of the clinical studies undertaken were conducted with
15 recombinant GH. GRF is considered as a second generation product destined to replace, in the near future, the use of GH in most instances. Accordingly, the use of GRF presents a number of advantages over the use of GH *per se*.

Physiological advantages

Growth hormone (GH) is secreted by the pituitary gland in a pulse
20 fashion. Since this rhythm of secretion is crucial for an optimal biological activity, the administration of GH to correspond to its natural mode of secretion is difficult to achieve. When GRF is administered in a continuous fashion as a slow releasing preparation or as an infusion, it increases GH secretion while respecting its pulsatility.

25 The recombinant GH which is presently commercialized is the 22 kDa form whereas GRF induces the synthesis and secretion from the pituitary gland of all the chemical isomers of GH which participate in a wider range of biological activities.

A treatment with GH results in a decreased capacity of the pituitary gland to secrete endogenous growth hormone, and the GH response to GRF is diminished after such a treatment. On the contrary, a treatment with GRF does not present this disadvantage, its trophic action on the pituitary gland increases this gland's secreting capacity in normal animals and in patients with somatotroph insufficiency.

Economical advantages

The production of GH by genetic engineering is very expensive for clinical use. In particular, there are risks of contamination of these commercial preparations with material from the bacterial strain used. These bacterial contaminants may be pyrogens or may result in immunogenic reactions in patients. The purification of the recombinant product is carried out by following a plurality of successive chromatography steps. The drastic purity criteria imposed by regulatory agencies necessitate multiple quality control steps.

On the other hand, the synthesis of GRF is of chemical nature. The synthesis is carried out in a solid phase and its purification is done in a single step using high performance liquid chromatography (HPLC). Also the quantity of GRF to be administered is much less than the quantity of GH for the same biological result.

Even with all these advantages, GRF is still not commercialized as a therapeutic agent to date, mainly because of its instability. The human GRF is a peptide of 44 amino acids of the following sequence:

25	Tyr	Ala	Asp	Ala	Ile	Phe	Thr	Asn	Ser	Tyr	Arg	Lys	Val	Leu	Gly	Gln
	1				5					10					15	
	Leu	Ser	Ala	Arg	Lys	Leu	Leu	Gln	Asp	Ile	Met	Ser	Arg	Gln	Gln	Gly
				20					25					30		
30	Glu	Ser	Asn	Gln	Glu	Arg	Gly	Ala	Arg	Ala	Arg	Leu-NH ₂				
			35					40					(SEQ ID NO:1).			

Gaudreau et al. (P. Gaudreau et al., 1992, *J. Med. Chem.*, **35**(10),:1864-1869) describe the affinity of acetyl-, 6-aminohexanoyl-, and

8-aminooctanoyl-GRF(1-29)NH₂ with the rat pituitary receptor. In this report, none of the fatty acid-GRF compounds tested exhibited a higher affinity than hGRF(1-29)NH₂ itself, and the authors concluded that "...modifications to increase the hydrophobic character at the N-terminus of hGRF(1-29)NH₂ do not constitute a suitable approach to increase receptor affinity."

Coy et al. (D.H. Cow et al., 1987, *J. Med. Chem.*, **30**:219-222) describe an acetyl-GRF peptide with an increased biological activity on a rat model, more particularly on a rat anesthetized with sodium pentobarbital. The *in vitro* GH response by cultured rat pituitary cells was also analyzed. However, these authors did not synthesize and test fatty acid-GRF analogs with a carbon chain longer than two (2) carbon atoms (acetyl group) added at the N-terminus region of the GRF and acetyl cannot be considered a hydrophobic group.

Up to now, most of the GRF analogs described (including those of Gaudreau et al. and those of Coy et al.) have been tested in rat models, either *in vitro* or *in vivo*. Since human and rat GRF(1-29)NH₂ are markedly different, the structure-activity relationships of GRF are different in both species. Therefore, it is not possible to extrapolate results obtained in rats to humans.

Accordingly, it is necessary to design GRF analogs with improved anabolic potency and having a prolonged activity. This increased potency could result from a resistance to serum degradation and/or from hyperagonistic properties.

It would be highly desirable to be provided with GRF analogs with increased anabolic potency.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide new biodegradable GRF analogs with improved biological potency and prolonged activity.

Another aim of the present invention is to provide GRF analogs
5 with increased anabolic potency and prolonged activity, i.e. capable to substantially elevate insulin-like growth factor I (IGF-I) levels when chronically administered in humans and animals.

Another aim of the present invention is to provide a means to
10 render any GRF analog more biologically potent and with a prolonged activity.

Another aim of the present invention is to provide a method of producing active GRF analogs with improved anabolic potency and prolonged activity.

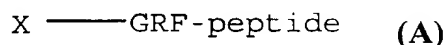
The present invention relates to the preparation of hydrophobic
15 GRF analogs. These chimeric analogs include a hydrophobic moiety (tail), and can be prepared, either by anchoring one or several hydrophobic tails to the GRF, or by substituting one or several amino-acids by a pseudomicellar residue in the chemical synthesis of GRF. The GRF analogs in accordance with the present invention are characterized in that:

- 20 a) These analogs possess an enhanced biological activity; specifically, they are able to markedly increase GH and IGF-I blood levels when administered in an animal model closely related to human. This characteristic is particularly advantageous in that it results in a reduced dosage of an hyperactive compound being administered to
25 the patient, thus improving treatment efficacy and reducing treatment costs.
- b) Both natural amino acid and hydrophobic substances, such as fatty acids, are used for the chemical synthesis of the GRF analogs.

- c) They present a high biological activity at infinitely small dosages.
- d) They remain active for a prolonged period of time, with a high biological activity.

The use of fatty bodies in accordance with the present invention results in GRF analogs which overcome all the drawbacks of the prior art. The GRF analogs of the present invention exhibit improved anabolic potency with a reduced dosage and have a prolonged activity. Furthermore, the present invention deals with GRF and any of its analogs, truncated or substituted.

In accordance with the present invention there is provided a hydrophobic GRF analog of formula A:



wherein;

the GRF peptide is a peptide of formula B;

A1-A2-Asp-Ala-Ile-Phe-Thr-A8-Ser-Tyr-Arg-Lys-A13-Leu-A15-Gln-Leu-A18-Ala-Arg-Lys-Leu-Leu-A24-A25-Ile-A27-A28-Arg-A30-R₀ (B)

wherein,

- A1** is Tyr or His;
- A2** is Val or Ala;
- A8** is Asn or Ser;
- A13** is Val or Ile;
- A15** is Ala or Gly;
- A18** is Ser or Tyr;
- A24** is Gln or His;
- A25** is Asp or Glu;
- A27** is Met, Ile or Nle;
- A28** is Ser or Asn;
- A30** is a bond or any amino acid sequence of 1 up to 15 residues;
- R₀** is NH₂ or NH-(CH₂)_n-CONH₂, with n=1 to 12 and;
- X** is a hydrophobic tail anchored via an amide bond to the N-terminus of the peptide and said hydrophobic tail defining a backbone of 5 to 7 atoms;

wherein said backbone can be substituted by C₁₋₆ alkyl, C₃₋₆ cycloalkyl, or C₆₋₁₂ aryl;

and comprises at least one rigidifying moiety connected to at least two atoms of the backbone;

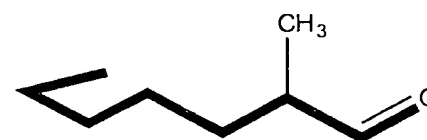
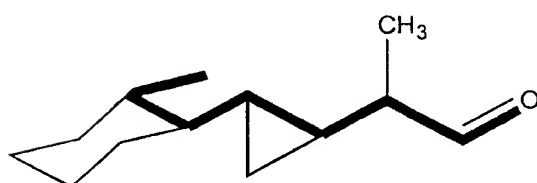
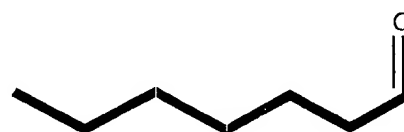
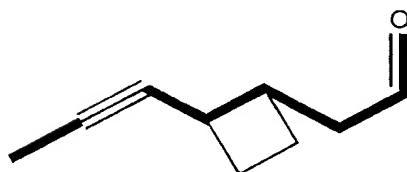
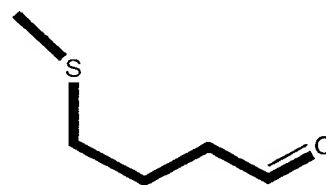
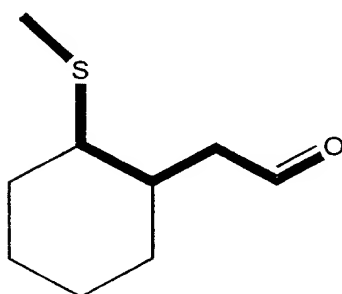
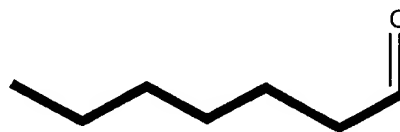
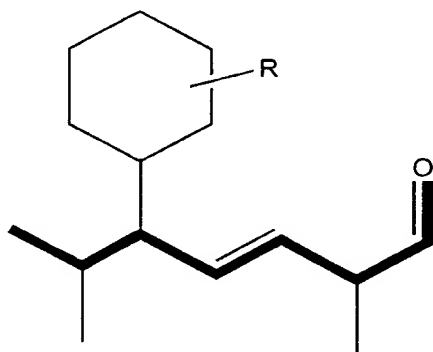
- 5 said moiety selected from the group consisting of
double bond, triple bond, saturated or unsaturated
C₃₋₉ cycloalkyl, and C₆₋₁₂ aryl.

- By the term rigidifying moiety is meant a moiety that will confer rigidity to the hydrophobic tail. The rigidifying moiety connects at least two
10 atoms which are part of the backbone of the hydrophobic tail. For example, the backbone of the following hydrophic tail is as follows:

09786639 063794
T02390 06998269

Tail

Backbone



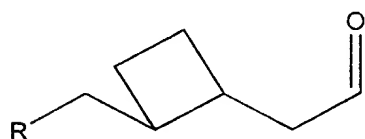
Preferably, the backbone is substituted with one rigidifying moiety selected from the group consisting of double bond, triple bond, saturated C₃₋₇ cycloalkyl and C₆ aryl.

Also preferably, the backbone is substituted with 2 rigidifying moieties which are independently selected from the group consisting of double bond and saturated or unsaturated C₃₋₉ cycloalkyl.

More preferably, the backbone is substituted with 2 rigidifying moieties which are independently selected from the group consisting of double bond, triple bond, saturated C₃₋₇ cycloalkyl and C₆ aryl.

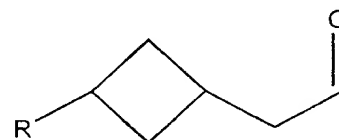
In an alternative embodiment, the backbone is substituted one
 5 rigidifying moiety selected from the group consisting of double bond, triple bond, saturated C₃₋₇ cycloalkyl and C₆ aryl, which are located at the 3,4-positions, the 3,5-positions or the 3,6-positions of the backbone.

Preferably, the hydrophobic tail is selected from the group consisting of:



3 (R = H or CH₃ or CH₂CH₃)

cis or *trans*, both as racemic mixtures or pure enantiomeric pairs.

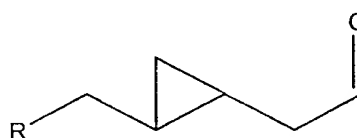


4 (R = H or CH₃ or CH₂CH₃)

cis or *trans*, (when R ≠ H)
 both as racemic mixtures or pure enantiomeric pairs.

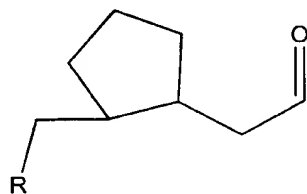


1 (R = H or CH₃ or CH₂CH₃)

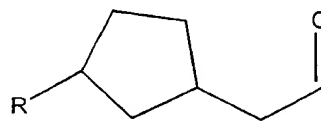


2 (R = H or CH₃ or CH₂CH₃)

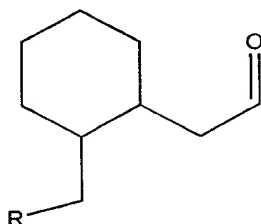
cis or *trans*, both as racemic mixtures or pure enantiomeric pairs.

5 (R = H or CH₃ or CH₂CH₃)

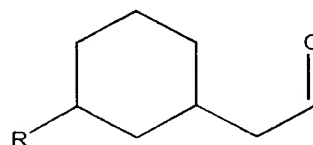
cis or *trans*, both as racemic mixtures or pure enantiomeric pairs.

6 (R = H or CH₃ or CH₂CH₃)

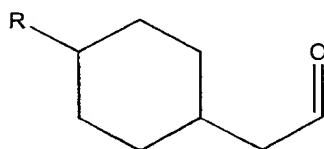
cis or *trans*, (when R ≠ H) both as racemic mixtures or pure enantiomeric pairs

7 (R = H or CH₃ or CH₂CH₃)

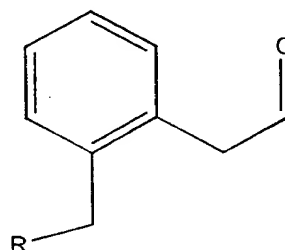
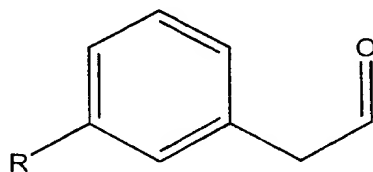
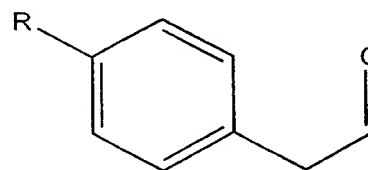
cis or *trans*, both as racemic mixtures or pure enantiomeric pairs.

8 (R = H or CH₃ or CH₂CH₃)

cis or *trans*, (when R ≠ H) both as racemic mixtures or pure enantiomeric pairs

9 (R = H or CH₃)

cis or *trans*, (when R ≠ H) both as racemic mixtures or pure enantiomeric pairs

10 (R = H or CH₃ or CH₂CH₃)11 (R = H or CH₃ or CH₂CH₃)12 (R = H or CH₃)

In accordance with the present invention, there is provided a method of increasing the level of growth hormone in a patient which

In accordance with the present invention, there is provided a method for the diagnosis of growth hormone deficiencies in patients, which comprises administering to said patient a GRF analog of the present invention and measuring the growth hormone response.

In accordance with the present invention, there is provided a method for the treatment of pituitary dwarfism or growth retardation in a patient, which comprises administering to said patient an effective amount of a GRF analog of the present invention.

In accordance with the present invention, there is provided a method for the treatment of wound or bone healing in a patient, which comprises administering to said patient an effective amount of a GRF analog of the present invention.

15 In accordance with the present invention, there is provided a method for the treatment of osteoporosis in a patient, which comprises administering to said patient an effective amount of a GRF analog of the present invention.

In accordance with the present invention, there is provided a
20 method for improving protein anabolism (including protein sparing effect) in
human or animal, which comprises administering to said human or animal
an effective amount of a GRF analog of the present invention.

In accordance with the present invention, there is provided a method for inducing a lipolytic effect in human or animal afflicted with clinical obesity, which comprises administering to said human or animal an effective amount of a GRF analog of the present invention.

In accordance with the present invention, there is provided a method for the overall upgrading of somatotroph function in human or

animal, which comprises administering to said human or animal an effective amount of a GRF analog of the present invention.

In the present invention the amino acids are identified by the conventional three-letter abbreviations as indicated below, which are as
5 generally accepted in the peptide art as recommended by the IUPAC-IUB commission in biochemical nomenclature:

Alanine	Ala
Arginine	Arg
Asparagine	Asn
Aspartic Acid	Asp
Cysteine	Cys
Glutamic Acid	Glu
Glycine	Gly
Histidine	His
Leucine	Leu
Lysine	Lys
Methionine	Met
Ornithine	Orn
Phenylalanine	Phe
Proline	Pro
Serine	Ser
Threonine	Thr
Tryptophane	Trp
Tyrosine	Tyr
D-Tyrosine	Tyr
Valine	Val

The term "natural amino acid" means an amino acid which occurs in nature or which is incorporated as an amino acid residue in a naturally

occurring peptide. In addition, the abbreviation Nle is intended to mean Norleucine.

Other abbreviations used are:

	TFA	Trifluoroacetic acid;
5	HOBt	1-Hydroxybenzotriazole;
	DIC	Diisopropylcarbodiimide;
	DMF	Dimethylformamide;
	Pip	Piperidine;
	DMAP	4-dimethylaminopyridine;
10	Boc	t-butyloxycarbonyl;
	Fmoc	Fluorenylmethyloxycarbonyl;
	BOP	Benzotriazo-1-yloxytris (dimethylamino) phosphonium hexafluorophosphate;
	Me	Methyl;
15	HF	Hydrofluoric acid;
	NEt ₃	Triethylamine; and
	TEAP	Triethylammonium phosphate (buffer).

All the peptide sequences set out herein are written according to the generally accepted convention whereby the N-terminal amino acid is on the left and the C-terminal amino acid is on the right.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph of the effect of subcutaneously injected hGRF(1-29)NH₂ analogs on pig serum IGF-1;

25 Fig. 2 is a curve of the effect of one intravenous injection of (4µg/kg) hGRF(1-29)NH₂ and (4µg/kg) (Hexenoyl trans-3)⁰ hGRF (1-29)NH₂ (TT-01024) + analog on pig serum GH;

Fig. 3 is a graph showing the effect of various doses of hGRF(1-29)NH₂ vs [hexenoyl trans-3]^o hGRF(1-29)NH₂ (TT-01024) on the GH area under the curve over 300 minutes following I.V. administration (**P<0.01 and ***P<0.001 when compared to the basal period --60 to 0 min-);

Fig. 4 is a curve of the effect of one subcutaneous injection of 5µg/kg hGRF(1-29)NH₂ and (5µg/kg) (Hexenoyl trans-3)^o hGRF (1-29)NH₂ analog on pig serum GH;

Fig. 5 is a graph showing the effect of various doses of hGRF(1-29)NH₂ vs [Hexenoyl trans-3]^o hGRF(1-29)NH₂ (TT-01024) on the GH area under the curve over 420 minutes following S.C. administration (**P<0.01 and ***P<0.001 when compared to the basal period --60 to 0 min-);

Fig. 6A to 6C illustrate examples of specific synthesis of the X-portion of GRF analogs with preferred radicals R in accordance with the present invention; and

Fig. 7A and 7B illustrate the effect of 8 different hGRF(1-44)NH₂ analogs of the present invention on IGF-1 levels in pigs.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the use of fatty bodies, namely pseudomicellar residues and/or hydrophobic tails, to produce a new family of highly potent, chimeric fatty body-GRF analogs.

In accordance with the present invention, the fatty body-GRF analogs can be chemically synthesized by anchoring one or several hydrophobic tails at the N- terminal portion of GRF or one of its analogs.

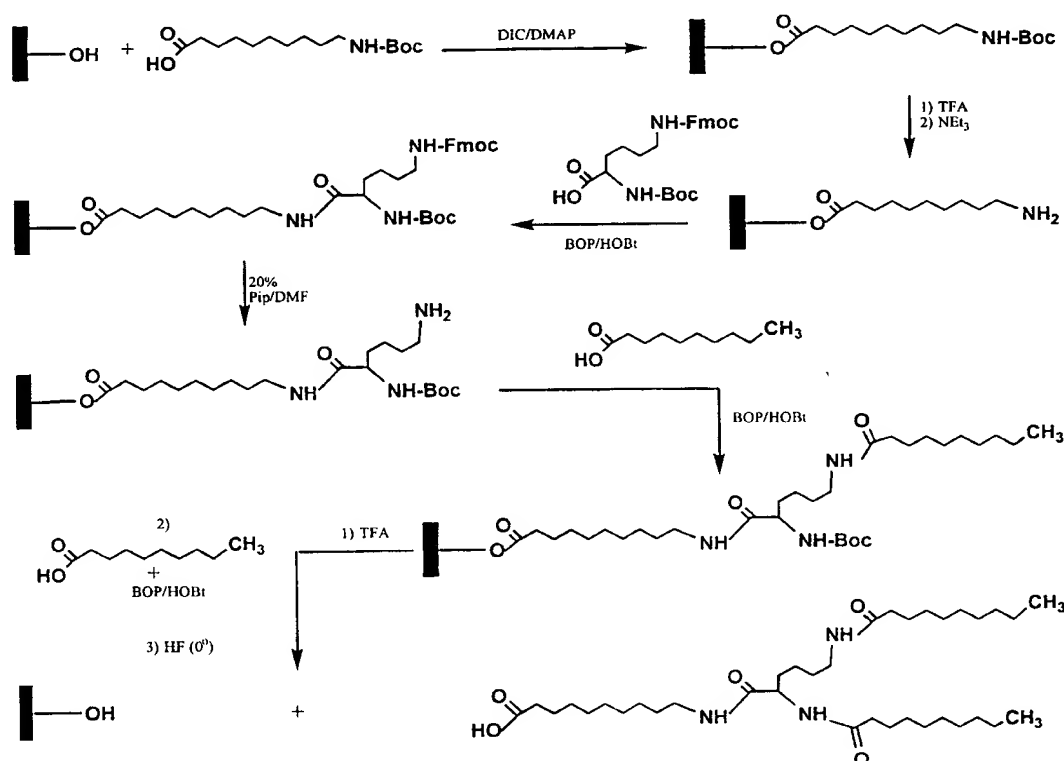
For a better carrying out of the chemical anchoring reaction, hydrophobic functionalized under the acid form are preferably used. In these conditions, the anchoring reaction is preferably effected in a solid phase (Merrifield R.B., 1963, *J. Am. Chem. Soc.*, **85**:2149; 1964, *J. Am.*

Chem. Soc., 86:304) using extremely active reagents such as for example Benzotriazole-1-yloxytris (dimethylamino) phosphonium hexafluorophosphate known in the prior art (B. Castro et al., 1975, *Tetrahedron letters*, Vol. 14:1219).

5 In the case where the hydrophobic tail to be anchored consists in a fatty acid, the activation in view of the anchoring may be carried out *in situ*. Depending on the synthesis strategies used, the peptide anchoring site is liberated just prior to the anchoring in traditional deprotection conditions (Gross et Meienhofer, 1981, *The peptides*, vol. 3, Academic press: pages 1-
10 341). The hydrophobic tail (Ht) is then condensed with the anchoring agent in organic solvents such as an ether (tetrahydrofuranne), an aliphatic halogenated solvent (dichloromethane), a nitrile (acetonitrile) or an amide (dimethylformamide).

With respect to the anchoring dynamic, the preferred working
15 temperatures are between 20 and 60°C. The anchoring reaction time when hydrophobic tail used are more and more hydrophobic, varies inversely with temperature, but varies between 0.1 and 24 hours.

As an illustrative example, the triacyl lysine synthesis as set forth below illustrates in a schematic manner the whole of the anchoring principle
20 of a hydrophobic fatty acid tail.



General GRF analogs synthesis steps were carried out by solid-phase methodology on a 9050TM plus peptide synthesizer (Millipore Corporation, Milford, MA) using Fmoc strategy and synthesis cycles supplied by Millipore. Fmoc amino acids were supplied by Bachem -

5 California and other commercial sources. Sequential Fmoc chemistry using BOP/HOBt as coupling methodology were applied to the starting Fmoc-Pal-PEG resin (Millipore, catalog number: GEN 913383) for the production of C-terminal carboxamides. Fmoc deprotections were accomplished with

10 piperidine 20% solution in DMF. After synthesis completion, the resin was well washed with DMF and ether prior to drying. Final cleavages of side chain protecting groups and peptide-resin bonds were performed using Millipore supplied procedure consisting of the following mixture: TFA, water, phenol, triisopropylsilane (88:5:5:2). Peptides were then precipitated

15 and washed with ether prior to drying. Reverse phase HPLC purification (buffer A: TEAP 2.5; buffer B: 80% CH₃CN in A) using a waters prep

4000, absorbance 214nm, detector model 486, flow rate 50ml/min.; linear gradient generally from 25 to 60%B in 105 min.) followed by a desalting step (buffer C:0.1% TFA in H₂O; buffer D:0.1% TFA in CH₃CH/H₂O 80:20) afforded peptides in yields amounting from 10 to 30% with
5 homogeneity greater than 97% as estimated by HPLC (millennium/photodiode array detection).

In accordance with the present invention, the pig was selected as a test specie, since it is a valuable preclinical model for the development of GRF analogs. Indeed, human and porcine GRF(1-29)NH₂ share a 100%
10 homology of structure, and the physiological pattern of GH secretion is almost identical in both species.

Moreover, the potency of the GRF analogs was assessed as their ability to significantly increase IGF-I blood levels rather than their acute GH releasing potency. Indeed, it is known that the anabolic and healing effects
15 of GH or GRF induced GH are mediated by an increase in IGF-I synthesis and secretion. Therefore, the measurement of GRF induced IGF-I elevation is the best indicator of the treatment efficacy.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the
20 invention rather than to limit its scope.

EXAMPLE I

25 **Effect of repeated administrations of [BUTYRYL⁰], [OCTANOYL⁰]-, [HEXANOYL⁰]-[HEXANOYL³⁰], [HEXANOYL^{0,30}], HGRF(1-29)NH₂ and [HEXANOYL⁰] HGRF(1-44)NH₂ VS hGRF(1-29)NH₂ on serum IGF-I levels in pigs**

The objective of these experiments was to assess the potential of the GRF analogs as anabolic agents. It is known that GH or GRF-induced
30 GH secretion exert their anabolic effect via an increase in insulin-like growth factor I (IGF-I) synthesis and secretion, that result in elevated levels

of circulating IGF-I. It has been previously demonstrated that the intensity of the anabolic response to a GRF analog treatment is proportional to the increase in IGF-I levels in pigs (Dubreuil P. et al., 1990, *J. Anim. Sci.*, 68:1254-1268).

- 5 Therefore, in order to investigate the anabolic potency of the fatty acid-GRF analogs, their ability to increase IGF-I levels following repeated S.C. administrations in pig was evaluated.

Experiment 1

26 Landrace x Yorkshire castrated male pigs (40-45kg BW) were
10 randomly distributed into 4 experimental groups:

- 1- hGRF(1-29)NH₂ (20µg/kg, n=7)
- 2- [octanoyl⁰] hGRF(1-29)NH₂ (20µg/kg, n=6)
- 3- [hexanoyl⁰] hGRF(1-29)NH₂ (20µg/kg, n=6)
- 4- [butyryl⁰] hGRF(1-29)NH₂ (20µg/kg, n=7)

15 Each animal was injected BID (twice a day) subcutaneously for 4 consecutive days. One blood sample was collected each morning prior to the first injection of the day, and the day after the last injection, for IGF-I measurement.

Experiment 2

20 40 Landrace x Yorkshire castrated male pigs (40-45 kg BW) were randomly distributed into 5 experimental groups:

- 1- saline (n=8)
- 2- hGRF(1-29)NH₂ (40µg/kg, n=8)
- 3- [hexanoyl⁰] hGRF(1-29)NH₂ (10µg/kg, n=8)
- 25 4- [hexanoyl⁰] hGRF(1-29)NH₂ (20µg/kg, n=8)
- 5- [hexanoyl⁰] hGRF(1-29)NH₂ (40µg/kg, n=8)

Each animal was injected BID (twice a day) subcutaneously for 5 consecutive days. One blood sample was collected each morning prior to

the first injection of the day, and the day after the last injection, for IGF-I measurement.

Experiment 3

48 Landrace x Yorkshire castrated male pigs (40-45 kg BW) were
5 randomly distributed into 6 experimental groups:

- 1- Saline (n=8)
- 2- hGRF(1-44)NH₂ (30µg/kg, n=8)
- 3- [hexanoyl⁰]hGRF(1-44)NH₂ (30µg/kg, n=8)
- 4- [hexanoyl⁰]hGRF(1-29)NH₂ (20µg/kg, n=8)
- 10 5- [hexanoyl³⁰]hGRF(1-29)NH₂ (20µg/kg, n=8)
- 6- [hexanoyl^{0, 30}]hGRF(1-29)NH₂ (20µg/kg, n=8)

The selected doses were 30µg/kg for hGRF(1-44)NH₂ analogs and 20µg/kg for hGRF(1-29)NH₂ analogs, which give identical doses on a molar basis. Each animal was injected BID (twice a day) subcutaneously for
15 5 consecutive days. One blood sample was collected each morning prior to the first injection of the day, and the day after the last injection, for IGF-I measurements.

IGF-I measurements

IGF-I levels were measured in pig serum by double antibody
20 radioimmunoassay after formic acid-acetone extraction, as previously described (Abribat T. et al., 1993, *J. Endocrinol.*, 39:583-589). The extraction prior to radioimmunoassay is a necessary step to remove endogenous IGF-binding proteins.

Statistical analysis

25 In both experiments, the IGF-I data were analyzed by a two way repeated measure analysis of variance, with day and treatment (GRF analog) as sources of variation. Multiple comparison procedures were there run

(Student-Newman Keuls method). A $P < 0.05$ was considered as statistically significant.

Results

Experiment 1

5 There were both a significant effect of day ($P=0.0004$) and a significant treatment x day interaction ($P=0.011$), indicating that the increase in IGF-I levels was dependent on the analog tested (Table 1). Blood samples for IGF-I measurements were collected daily prior to the first injection of compounds. Data are shown as mean \pm SEM of 6 to 7 values
10 per group.

Table 1

Effect of repeated SC injection (20 μ g/kg BID x 4 days) of GRF analogs on serum IGF-I levels

Treatment (BID, 20 μ g/kg SC)	Day 1 (pretreatment) (ng/ml)	Day 2 (ng/ml)	Day 3 (ng/ml)	Day 4 (ng/ml)	Day 5 (ng/ml)
hGRF(1-29)NH ₂	252 \pm 28	235 \pm 19	263 \pm 16	258 \pm 17	262 \pm 24
[octanoyl ⁰] hGRF(1-29)NH ₂	316 \pm 22	287 \pm 20	301 \pm 37	301 \pm 37	318 \pm 39
[hexanoyl ⁰] hGRF(1-29)NH ₂	248 \pm 20	281 \pm 28	299 \pm 26	319 \pm 22 ^a	342 \pm 21 ^{a,b}
[butyryl ⁰] hGRF(1-29)NH ₂	278 \pm 20	281 \pm 24	302 \pm 26	289 \pm 26	293 \pm 23

15 Treatment $P=0.42$

Day $P=0.0004$

Treatment x Day $P=0.011$

^a $P < 0.05$ when compared to day 1

^b $P < 0.05$ when compared to day 2

20

Multiple comparisons revealed that only [hexanoyl⁰] hGRF(1-29)NH₂ elicited an increase in IGF-I levels, which was significant on days 4

(29%, $P < 0.05$) and 5 (38%, $P < 0.05$). Human GRF(1-29)NH₂ had no effect on IGF-I levels at the dose tested.

Experiment 2

There were both a significant effect of day ($P < 0.0001$) and a significant treatment x day interaction ($P < 0.0001$), indicating that the increase in IGF-I levels was dependent on the analog tested (Table 2). Blood samples for IGF-I measurements were collected daily prior to the first injection of the day. Data are shown as mean \pm SEM of 8 values per group.

Table 2

Dose-related effect of repeated SC injection (BID x 5 days) of GRF analogs on serum IGF-I levels

Treatment BID, SC	Day 1 (pretreat- ment) (ng/ml)	Day 2 (ng/ml)	Day 3 (ng/ml)	Day 4 (ng/ml)	Day 5 (ng/ml)	Day 6 (ng/ml)
saline	282 \pm 33	266 \pm 30	281 \pm 34	293 \pm 30	287 \pm 32	289 \pm 33
hGRF(1-29)NH ₂ (40 μ g/kg)	244 \pm 24	243 \pm 16	267 \pm 20	275 \pm 27	267 \pm 17	256 \pm 15
[hexanoyl ⁰] hGRF (1-29)NH ₂ (10 μ g/kg)	303 \pm 31	327 \pm 20	337 \pm 25	338 \pm 25	366 \pm 37 ^a	350 \pm 34 ^a
[hexanoyl ⁰] hGRF (1-29)NH ₂ (20 μ g/kg)	302 \pm 38	341 \pm 37	368 \pm 43 ^a	362 \pm 40 ^a	362 \pm 45 ^a	368 \pm 57 ^a
[hexanoyl ⁰] hGRF (1-29)NH ₂ (40 μ g/kg)	252 \pm 35	275 \pm 32	319 \pm 31 ^a	354 \pm 41 ^{a,b}	350 \pm 34 ^{a,b}	374 \pm 33 ^{a,b,c}

Treatment $P=0.23$; Day $P=0.0001$

Treatment x Day $P=0.0001$

^a $P < 0.05$ when compared to day 1

^b $P < 0.05$ when compared to day 2

^c $P < 0.05$ when compared to day 3

Multiple comparisons revealed that all three tested doses of [hexanoyl⁰] hGRF(1-29)NH₂ increased IGF-I levels. At 10 μ g/kg, IGF-I levels were significantly increased at days 5 and 6 (16 to 21%, $P < 0.05$). At

20µg/kg, they were increased at days 3, 4, 5 and 6 (20 to 22%, $P < 0.05$). At 40µg/kg, they were increased at days 3, 4, 5 and 6 (27 to 48%, $P < 0.05$). The serum IGF-I levels remained stable in saline - and hGRF(1-29)NH₂ - treated pigs.

- 5 Finally, a regression analysis revealed that the increase in IGF-I concentrations from day 1 to day 6 was dependent on the dose of [hexanoyl⁰] hGRF(1-29)NH₂ (Δ IGF-I = $11.9 + (2.77 * \text{dose})$; $r = 0.68$, $P < 0.0001$).

Experiment 3

- 10 There were both a significant effect of day ($P < 0.0001$) and a significant treatment x day interaction ($P < 0.0001$), indicating that the increase in IGF-I levels was dependent on the analog tested (Table IV). Multiple comparison revealed that analogs with an hexanoyl function branched at the N-terminal region of GRF were highly potent:

- 15 - [hexanoyl⁰] hGRF(1-29)NH₂ significantly increased IGF-I levels on days 5 and 6 (by 28% and 31%, $P < 0.05$)
 - [hexanoyl^{0, 30}] hGRF(1-29)NH₂ significantly increased IGF-I levels on days 4, 5 and 6 (by 32%, 35% and 43%, $P < 0.05$)
 - [hexanoyl⁰] hGRF(1-44)NH₂ significantly increased IGF-I levels
20 on days 3, 4, 5 and 6 (by 41%, 54%, 50% and 61%, $P < 0.05$)

- As previously observed for hGRF(1-29)NH₂ (experiments 1 and 2), the full length hGRF(1-44)NH₂ had little or no effect on IGF-I levels (except for a significant effect on day 5, which was not sustained on day 6). Finally, the anchoring of an hexanoyl function at the C-terminal region of
25 hGRF(1-29)NH₂ yielded an analog with increased potency when compared to hGRF(1-29)NH₂ (21% increased in IGF-I levels on day 6, $P < 0.05$), but less potent than [hexanoyl⁰]hGRF(1-29)NH₂.

Human GRF(1-29)NH₂ and hGRF(1-44)NH₂ were injected at 20µg/kg and 30µg/kg, respectively, in order to achieve equimolar concentrations. Data shown are mean ± SEM of 8 values per group.

Table 3

5 **Effect of multiple SC injections of GRF analogs (BID x 5 days) on serum IGF-I levels in growing pigs**

Treatment BID, SC	Day 1 (pretreat- ment) (ng/ml)	Day 2 (ng/ml)	Day 3 (ng/ml)	Day 4 (ng/ml)	Day 5 (ng/ml)	Day 6 (ng/ml)
saline	215 ± 21	215 ± 28	219 ± 25	226 ± 28	249 ± 30	234 ± 24
hGRF(1-44)NH ₂ (30µg/kg)	245 ± 21	254 ± 22	285 ± 26	297 ± 28	303 ± 26 ^a	296 ± 26
[hexanoyl ⁰] hGRF(1- 29)NH ₂ (20µg/kg)	272 ± 45	292 ± 52	292 ± 57	315 ± 57	347 ± 44 ^{a,b,c}	356 ± 44 ^{a,b,c}
[hexanoyl ³⁰] hGRF(1- 29)NH ₂ (20µg/kg)	297 ± 30	270 ± 25	287 ± 24	278 ± 18	276 ± 20	327 ± 24 ^b
[hexanoyl ^{0,30}] hGRF(1- 29)NH ₂ (20µg/kg)	205 ± 24	212 ± 26	253 ± 33	271 ± 36 ^{a,b}	277 ± 29 ^{a,b}	294 ± 26 ^{a,b}
[hexanoyl ⁰] hGRF(1- 44)NH ₂ (30µg/kg)	241 ± 30	290 ± 33	340 ± 41 ^a	372 ± 40 ^{a,b}	361 ± 46 ^{a,b}	388 ± 49 ^{a,b,c}

Treatment P=0.16

^a P < 0.05 when compared to day 1

Day P<0.0001

^b P < 0.05 when compared to day 2

Treatment x Day P<0.0001 ^c P < 0.05 when compared to day 3

10 **Conclusions**

Neither hGRF(1-29)NH₂ nor hGRF(1-44)NH₂ at doses ranging from 20 to 40 µg/kg were able to modulate IGF-I levels. However, the anchoring of fatty acid rendered GRF more potent and yielded analogs with markedly improved activity on IGF-I secretion. The anchoring of fatty acids was efficient in improving the anabolic potency of both hGRF(1-29)NH₂ and hGRF(1-44)NH₂. From the above results, it is concluded that the ideal fatty acid to use is hexanoic acid or any C6 fatty derivative, and that it

should be preferably anchored at the N-terminal region of GRF to yield maximally potent analogs.

EXAMPLE II

5 **Comparative effects of GRF analogs on IGF-I levels in pigs**

This was a 5-day treatment, twice a day S.C. administration of one single dose of each test article vs saline. This experiment was conducted to compare the efficacy of (Aminohexanoyl)₀ hGRF (1-29) NH₂, (Hexylformiate)₀ hGRF (1-29) NH₂, (Hexenoyl trans-2)₀ hGRF (1-29) NH₂, (Hexenoyl trans-3)₀ hGRF (1-29) NH₂ and (Muconoyl)₀ hGRF (1-29) NH₂ to that of (Hexanoyl)₀ hGRF (1-29) NH₂.

All tested compounds belong to the same family of GRF analogs: they are a combination of the natural GRF and natural fatty acids, designed to improve the activity of the molecule.

15	Identity of tested analogs:	<u>in saline</u>
	TT-01015 (Hexanoyl) ₀ hGRF (1-29) NH ₂	20 µg/kg
	TT-01021 (Aminohexanoyl) ₀ hGRF (1-29) NH ₂	20 µg/kg
	TT-01022 (Hexylformiate) ₀ hGRF (1-29) NH ₂	20 µg/kg
	TT-01023 (Hexenoyl trans-2) ₀ hGRF (1-29) NH ₂	20 µg/kg
20	TT-01024 (Hexenoyl trans-3) ₀ hGRF (1-29) NH ₂	20 µg/kg
	TT-01025 (Muconoyl) ₀ hGRF (1-29) NH ₂	20 µg/kg

Route and frequency of test article

ADMINISTRATION: Two daily subcutaneous injections.

TEST SYSTEM: Landrace x Yorkshire pigs.

25 ANIMAL DESCRIPTION: Fifty six (56) growing barrows pigs weighing 35 kg at the time of purchase.

RATION: Commercial feed concentrate (18% protein) offered ad libitum.

EXPERIMENTAL

DESIGN: Fifty six (56) pigs were randomly distributed into 7 experimental groups (n = 8 pigs per group). Each group received two daily S.C. administration of the following treatments (volume: 3 ml, S.C. injection).

group 1: saline 2 x/day

group 2: TT-01015 20 µg/kg 2 x/day

group 3: TT-01021 20 µg/kg 2 x/day

group 4: TT-01022 20 µg/kg 2 x/day

group 5: TT-01023 20 µg/kg 2 x/day

group 6: TT-01024 20 µg/kg 2 x/day

group 7: TT-01025 20 µg/kg 2 x/day

Treatments were administered from day 1 to 5. Immediately before the injections, one blood sample were collected from each animal, and additional blood samples were collected on day 6.

Blood samples were allowed to clot, serum was harvested by centrifugation and submitted to IGF-I assays.

Results are shown in Fig. 1 as D-IGF-I, which is defined as the increase in IGF-I levels from day 1 (pretreatment levels) to day 6 (after 5 days of GRFs administrations). Among all analog tested, only hexanoyl-, hexylformiate-, hexenoyl trans2- and hexenoyl trans3-hGRF(1-29)NH₂ significantly increased IGF-I levels over the 6-day study period, whereas aminohexanoyl- and muconoyl-hGRF(1-29)NH₂ did not. Since hGRF(1-29)NH₂ has been shown to be ineffective at the same dose in the same conditions in previous assays (see Example I), these results show that the addition of various C6 carbon chains at the N-terminus region of GRF increases its bioactivity.

EXAMPLE III

Intravenous GH-releasing potency of (Hexenoyl trans-3)₀ hGRF (1-29) NH₂ vs hGRF(1-29)NH₂ in pigs

5 This experiment was conducted to test the I.V. acute GH-releasing potency of (Hexenoyl trans-3)₀ hGRF (1-29) NH₂, a GRF analog, in a model physiologically close to human and to compare it to that of hGRF(1-29)NH₂.

(Hexenoyl trans-3)₀ hGRF (1-29) NH₂ is a combination of the natural hGRF(1-29)NH₂ and natural fatty acids. This study was a
10 multidose, single I.V. injection study.

Identity of tested analogs:

TT-01024	(Hexenoyl trans-3) ₀ hGRF (1-29) NH ₂	0.25 µg/kg
TT-01024	(Hexenoyl trans-3) ₀ hGRF (1-29) NH ₂	1 µg/kg
TT-01024	(Hexenoyl trans-3) ₀ hGRF (1-29) NH ₂	4 µg/kg
15	hGRF(1-29)NH ₂	0.25 µg/kg
	hGRF(1-29)NH ₂	1 µg/kg
	hGRF(1-29)NH ₂	4 µg/kg

Route and frequency of test article

ADMINISTRATION: intravenous acute injection.
20 TEST SYSTEM: Landrace x Yorkshire pigs.
ANIMAL DESCRIPTION: Fifty six (56) growing barrows pigs weighing 35 kg at the time of purchase.

RATION: Commercial feed concentrate (18% protein) offered ad libitum.

25 EXPERIMENTAL

DESIGN: Fifty (56) pigs (4 spare animals) were cannulated (a catheter surgically implanted in one jugular vein) within one week, before the study. On days 1 and 7,

cannulated animals were randomly distributed into 7 groups (n = 4 pigs per group).

group 1: saline

group 2: TT-01024 0.25 µg/kg

5 group 3: TT-01024 1 µg/kg

group 4: TT-01024 4 µg/kg

group 5: hGRF(1-29)NH₂ 0.25 µg/kg

group 6: hGRF(1-29)NH₂ 1 µg/kg

group 7: hGRF(1-29)NH₂ 4 µg/kg

10 Blood samples for pGH assay were collected every 20 min from 1 hour before to 5 hours after GRF injections, with additional samplings 10 and 30 min after injection (n = 21 samples). Blood samples are allowed to clot at +4°C. Serum will be harvested by centrifugation, stored at -20°C and submitted to pGH assays.

15 Results are illustrated in Figs. 2 and 3. As shown in Fig. 2, hGRF(1-29)NH₂ (4 µg/kg) induced a rapid GH release that was sustained for approximately 60 minutes following injection. In contrast, hexenoyl trans3-hGRF(1-29)NH₂ injected at the same dose increased GH levels over a longer period, approximately 260 minutes. In addition, the GH response in
20 the first 60 minutes was moderate, suggesting that this analog acts as a GRF, being processed in serum into native GRF in the minutes or hours following injection. As shown in Fig. 3, which presents the effects of various doses of GRF and the analog on the GH area under the curve (0 to 300 minutes following injection), hGRF(1-29)NH₂ produced a significant effect on GH
25 secretion at 4µg/kg, but not at 0.25 or 1 µg/kg, whereas hexenoyl trans3-hGRF(1-29)NH₂ elicited a significant response at all 3 doses tested. In conclusion, these results show that hexenoyl trans3-hGRF(1-29)NH₂ is a

GRF analog with increased potency on GH secretion, and suggest that it may act as a GRF, being protected from enzymatic degradation in serum.

EXAMPLE IV

5 **Subcutaneous GH-releasing potency of (Hexenoyl trans-3)₀ hGRF (1-29) NH₂ vs hGRF(1-29)NH₂ in pigs**

This experiment was conducted to test the S.C. acute GH-releasing potency of (Hexenoyl trans-3)₀ hGRF (1-29) NH₂, a GRF analog, in a model physiologically close to human and to compare it to that of hGRF(1-29)NH₂.

10 **Identity of tested analogs:**

TT-01024	(Hexenoyl trans-3) ₀ hGRF (1-29) NH ₂	0.31 µg/kg
TT-01024	(Hexenoyl trans-3) ₀ hGRF (1-29) NH ₂	1.25 µg/kg
TT-01024	(Hexenoyl trans-3) ₀ hGRF (1-29) NH ₂	5 µg/kg
TT-01024	(Hexenoyl trans-3) ₀ hGRF (1-29) NH ₂	20 µg/kg
15	hGRF(1-29)NH ₂	1.25 µg/kg
	hGRF(1-29)NH ₂	5 µg/kg
	hGRF(1-29)NH ₂	20 µg/kg

Route and frequency of test article

ADMINISTRATION: Subcutaneous acute injection.

20 TEST SYSTEM: Landrace x Yorkshire pigs.

ANIMAL DESCRIPTION: Sixty four (64) growing barrows pigs weighing 35 kg at the time of purchase.

RATION: Commercial feed concentrate (18% protein) offered ad libitum.

25

EXPERIMENTAL

DESIGN: Thirty six (36) pigs (4 spare animals) were cannulated (a catheter surgically implanted in one jugular vein) within one

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week, before the study. On days 1 and 7, cannulated animals were randomly distributed into 8 groups (n = 4 pigs per group).

group 1: saline

5 group 2: TT-01024 0.31 µg/kg

group 3: TT-01024 1.25 µg/kg

group 4: TT-01024 5 µg/kg

group 5: TT-01024 20 µg/kg

group 6: hGRF(1-29)NH₂ 1.25 µg/kg

10 group 7: hGRF(1-29)NH₂ 5 µg/kg

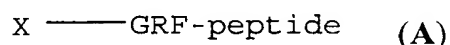
group 8: hGRF(1-29)NH₂ 20 µg/kg

Blood samples for pGH assay were collected every 20 min from 1 hour before to 7 hours after GRF injections, (n = 25 samples). Blood samples were allowed to clot at +4CC. Serum is harvested by centrifugation,
15 stored at -20°C and submitted to pGH assays.

Results are shown in Figs. 4 and 5. As shown in Fig. 4, the subcutaneous injection of 5 µg/kg hGRF(1-29)NH₂ induced a GH response in the first 60 minutes following administration, whereas the same injection of hexenoyl trans3-hGRF(1-29)NH₂ induced a GH response that was
20 sustained for 240 minutes. The Fig. 5 illustrates the effect of various doses of the GRFs tested on the GH area under the curve over the study period, i.e. from 0 to 420 minutes following injection. Over this period, hGRF(1-29)NH₂ did not induce any significant GH response at any of the tested doses, whereas hexenoyl trans3-hGRF(1-29)NH₂ elicited significant
25 increases of the GH AUC at 5 and 20 µg/kg. Altogether, these results suggest that hexenoyl trans3-hGRF(1-29)NH₂ is a highly potent GH secretagogue, even when subcutaneously administered.

EXAMPLE V

In accordance with a preferred embodiment of the present invention there is provided a hydrophobic GRF analog of formula A:



5 wherein;

the GRF peptide is a peptide of formula B;

A1-A2-Asp-Ala-Ile-Phe-Thr-A8-Ser-Tyr-Arg-Lys-A13-Leu-A15-Gln-Leu-A18-Ala-Arg-Lys-Leu-Leu-A24-A25-Ile-A27-A28-Arg-A30-R₀ (B)

10 wherein,

A1 is Tyr or His;

A2 is Val or Ala;

A8 is Asn or Ser;

A13 is Val or Ile;

15

A15 is Ala or Gly;

A18 is Ser or Tyr;

A24 is Gln or His;

A25 is Asp or Glu;

A27 is Met, Ile or Nle;

20

A28 is Ser or Asn;

A30 is a bond or any amino acid sequence of 1 to 15 residues;

R₀ is NH₂ or NH-(CH₂)_n-CONH₂, with n=1 to 12 and;

X is cis or trans CH₃-CH₂-CH=CH-CH₂-CO-, or

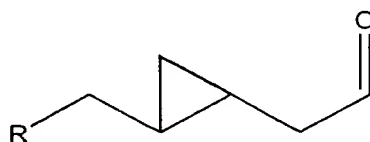
one element selected from a cis or a trans enantiomer or a racemic mixture

25 of:

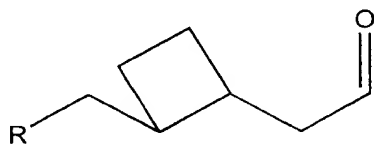


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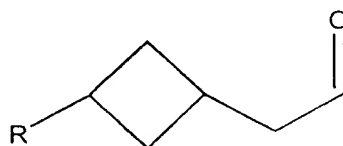
1 (R = H or CH₃ or CH₂CH₃)



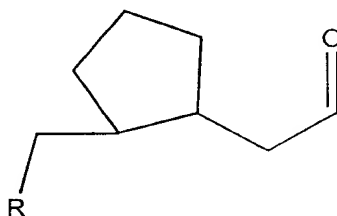
2 (R = H or CH₃ or CH₂CH₃)



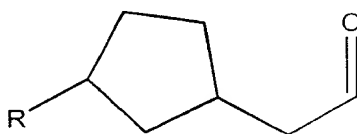
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3 (R = H or CH₃ or CH₂CH₃)

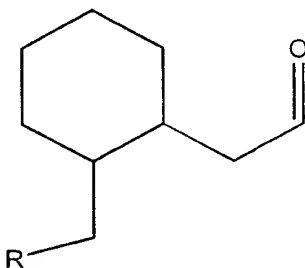
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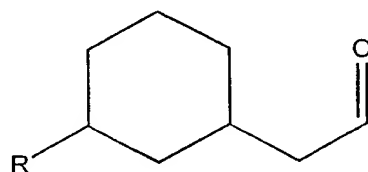
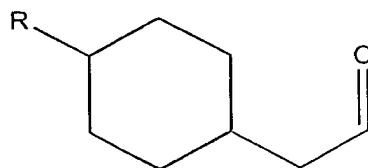
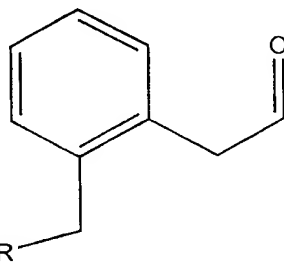
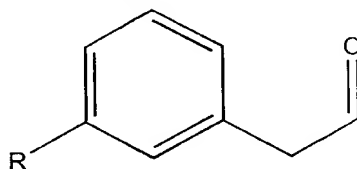
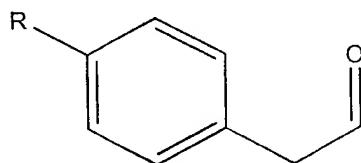
4 (R = H or CH₃ or CH₂CH₃)

15

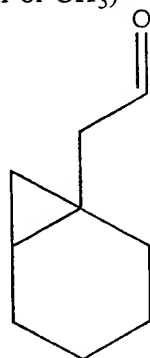
5 (R = H or CH₃ or CH₂CH₃)6 (R = H or CH₃ or CH₂CH₃)

20

7 (R = H or CH₃ or CH₂CH₃)

8 (R = H or CH₃ or CH₂CH₃)9 (R = H or CH₃)10 (R = H or CH₃ or CH₂CH₃)11 (R = H or CH₃ or CH₂CH₃)12 (R = H or CH₃)

and



13

wherein R is a hydrogen or a lower alkyl.

Fig. 6A to 6C illustrate examples of specific synthesis of the X-portion of GRF analogs with preferred radicals R in accordance with the present invention.

- 5 The acyl radicals X identified above with the numerals 10 (R=CH₃), and 11 (R=CH₃) are derived from the precursor carboxylic acids (X-OH) sold by Aldrich under catalog numbers T3,808-3 and T3,809-1.

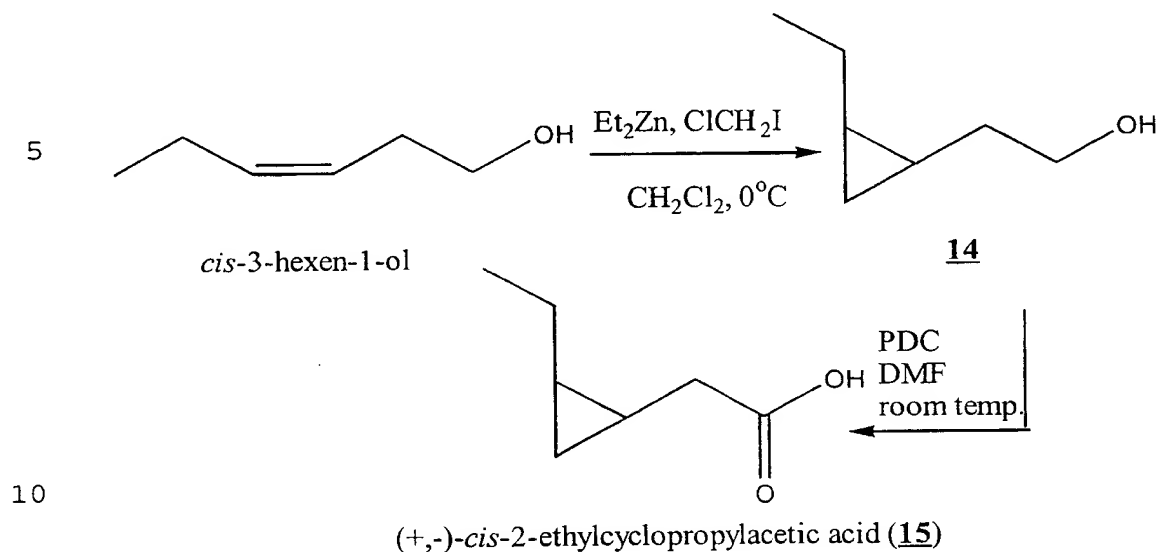
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EXAMPLE VI

Synthesis of (+,-)-cis-2-ethylcyclopropylacetic acid (15)

- Synthesis of (+,-)-cis-2-ethylcyclopropylacetic acid (15) was accomplished as illustrated in scheme 1. First the *cis*-3-hexen-1-ol was reacted with diethyl zinc in presence of chloriodomethane (Scott E. Denmark and James P. Edwards *J. Org. Chem.* **1991**, 56, 6974-6981) in
15 dichloromethane at 0°C to give the desired cyclopropyl alcohol 14 in 92% yield. Oxidation of the alcohol 14 with pyridinium dichromate (Corey, E. J. and Schmidt, G. *Tetrahedron Lett.* **1979**, 399-402) in dry DMF gave the (+,-)-cis-2-ethylcyclopropylacetic acid (15) in 66% yield.

Scheme 1



Preparation of (+,-)-*cis*-2-ethylcyclopropylacetic acid (15)

1. Preparation of cyclopropyl alcohol 14

In a 250 ml, one necked flask, a solution of Et_2Zn (4.0 ml; 40 mmol; 2 eq.) in dry dichloromethane (70 ml) was cooled to 0°C , and a solution of ClCH_2I (5.8 ml; 80 mmol; 4 eq.) was added *via* syringe. The solution was stirred for 5 min at 0°C , during which time a precipitate was formed, and a solution of *cis*-3-hexen-1-ol (2.0 g, 20 mmol) in CH_2Cl_2 (32 ml) was added *via* cannula. The reaction mixture was stirred for 90 min at 0°C and quenched with a saturated aqueous solution of NH_4Cl (100ml). The solution was then allowed to warm to room temperature, stirred vigorously for 10 min, and extracted with dichloromethane (3 x 60 ml). The extracts were washed with H_2O (1 x 20 ml) and brine (1 x 20 ml), combined, dried (MgSO_4), and concentrated under reduced pressure. The residue was purified by silica gel chromatography (hexane/ AcOEt , 8/2) to afford 2.10 g (92.3%) of the desired product 14 as a clear, colorless liquid.

$R_f = 0.22$ (hexane/ AcOEt , 8/2)

Characterization : Nmr (^1H , ^{13}C) , ir, ms

2. Preparation of (+,-)-*cis*-2-ethylcyclopropylacetic acid (15)

Alcohol 14 (2.30 g; 19.8 mmol) was dissolved in dry DMF (96 ml) and pyridinium dichromate (PDC) (3.5 eq., 26 g) was added in one
5 portion. The reaction mixture was stirred overnight at room temperature and then poured into 300 ml of water and extracted with (4 x 80 ml) of ether. The organic layers were combined, washed with brine and concentrated under reduced pressure. The residue was then dissolved in chloroform (30 ml) and the solution was extracted two times with 25 ml of a 10% aqueous
10 solution of NaOH. Combined aqueous phases were then extracted with chloroform (2 x 25 ml) and acidified with 10% aqueous HCl followed by an extraction with ether (4 x 50 ml). The combined organic phases were dried over MgSO_4 , filtered and concentrated under reduced pressure to give the desired product (+,-)-*cis*-2-ethylcyclopropylacetic acid (15) as a colorless oil
15 (1.7 g, 66%)

Characterization : Nmr (^1H , ^{13}C) , ir, ms

EXAMPLE VII

20 Synthesis of (1*R*,2*S*)-2-ethylcyclopropylacetic acid (22) and (1*R*,2*R*)-2-ethylcyclopropylacetic acid (24)

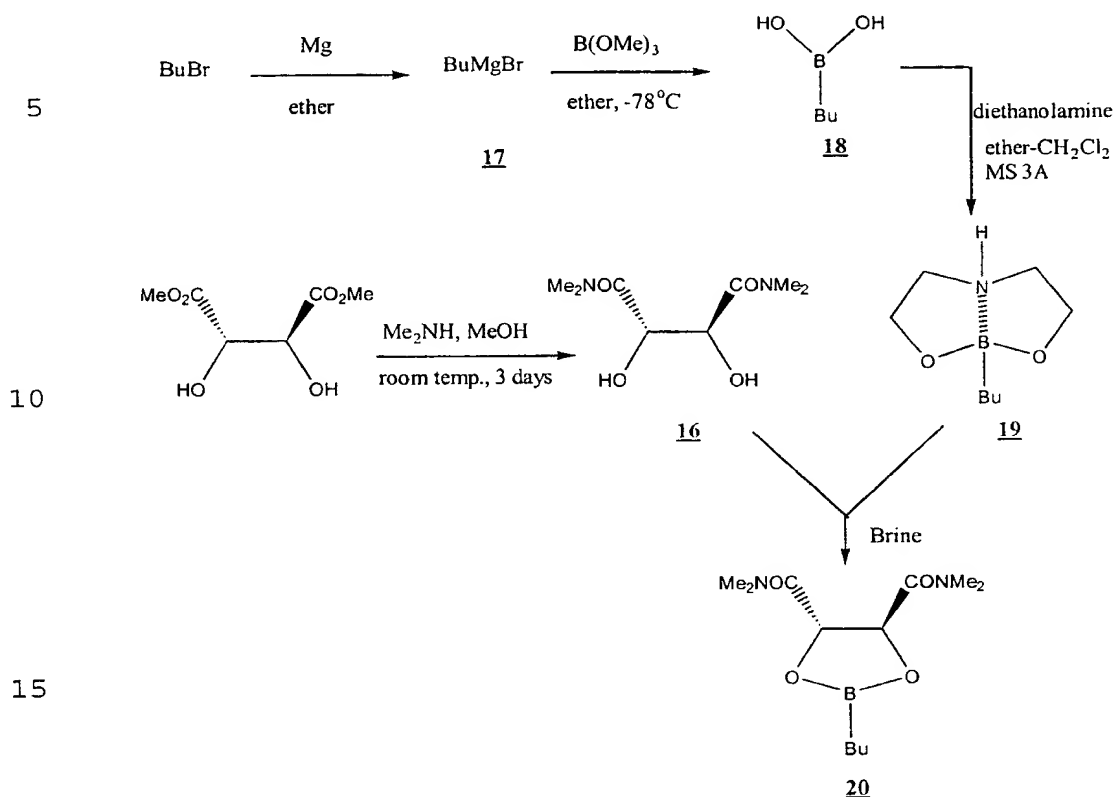
The synthesis of the optically pure *cis* and *trans* acids (1*R*,2*R*)-2-ethylcyclopropylacetic acid (24) and (1*R*,2*S*)-2-ethyl-cyclopropylacetic acid (22) was accomplished according to the protocol of Charette.(Charette, A. B.; Juteau, H.; Lebel, H.; and Molinaro C. *J. Am. Chem. Soc.* **1998**, *120*,
25 11943-11952) To achieve this, the chiral dioxaborolane ligand 20 was required.

1. Synthesis of the dioxaborolane ligand 20

The synthesis (scheme 2) began with the preparation of butyl boronic acid 18 via the treatment of butyl magnesium bromide with trimethyl borate followed by acidic hydrolysis. To avoid the dehydration of butyl boronic acid and its transformation into boroxine, it was converted to its diethanolamine complex 19. (*R,R*)-(+)-N,N,N,'N,'-tetramethyl tartaric acid diamide 16 was easily prepared using Seebach's procedure (Seebach, D.; Kalinowski, H.-O.; Langer, W.; Wilka, E.-M. *Organic Syntheses*; Wiley : New York, 1990; Coll. Vol. VII, pp 41-50) by condensing (*R,R*)-(+)-dimethyl tartaric acid with dimethyl amine.

The diethanolamine complex 19, when treated with a slight excess of (*R,R*)-(+)-N,N,N,'N,'-tetramethyl tartaric acid diamide (16) in a biphasic medium, reacted to give the desired chiral dioxaborolane ligand 20 in 93% yield.

Scheme 2

2. Synthesis of (1*R*,2*S*)-2-ethylcyclopropylacetic acid (22)

20

Once the dioxaborolane ligand 20 was in hand, we proceeded with the synthesis of (1*R*,2*S*)-2-ethylcyclopropylacetic acid (22) as shown in scheme 3. Thus, the homoallylic alcohol *trans* -3-hexen-1-ol was added to a mixture of the dioxaborolane 20 ligand and the zinc reagent ($\text{Zn}(\text{CH}_2\text{I})_2 \cdot \text{DME}$) (obtained from the mixture of Et_2Zn , CH_2I_2 and DME) in

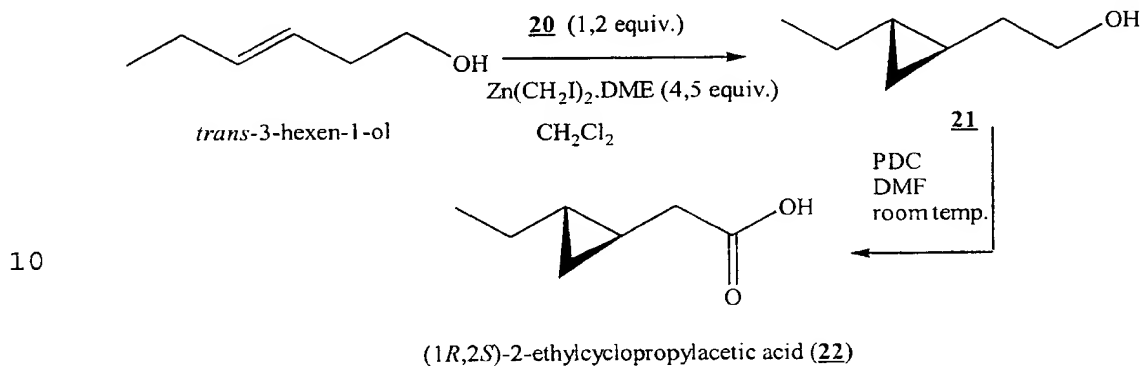
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dichloromethane at -10°C . The homogenous mixture was then warmed to room temperature and stirred overnight. Non-oxidative work-up afforded the desired product 21 in 79% yield, after a Kugelrohr distillation. Finally,

pyridinium dichromate (PDC) oxidation of alcohol **21** gave the (1*R*,2*S*)-2-ethylcyclopropylacetic acid (**22**) in 39.7% yield (scheme 3).

Scheme 3

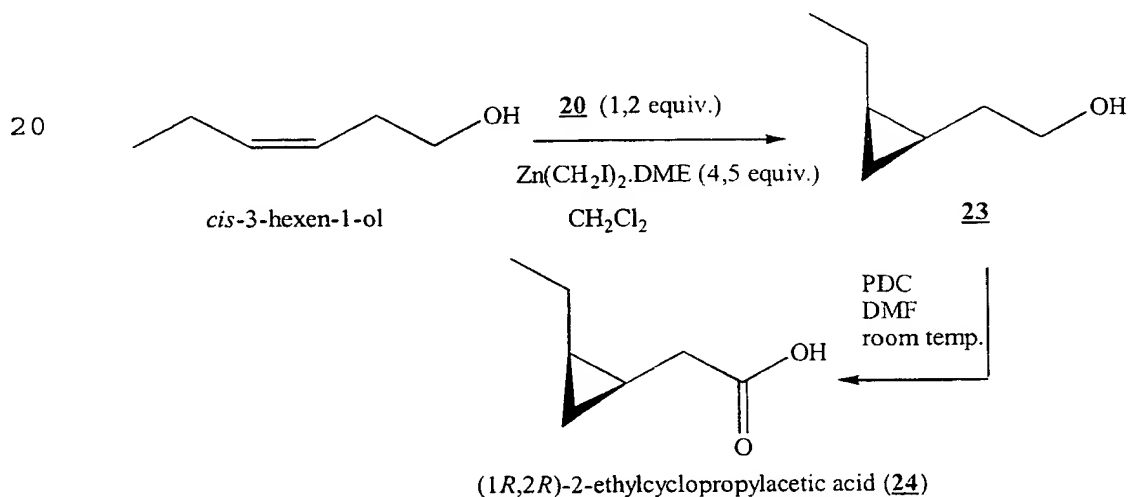
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3.Synthesis of (1*R*,2*R*)-2-ethylcyclopropylacetic acid (**24**)

The synthesis of optically active (1*R*,2*R*)-2-ethylcyclopropylacetic acid acid (**24**) (scheme 4), was accomplished by using the same method used to produce (1*R*,2*S*)-2-ethylcyclopropylacetic acid (**22**).

Scheme 4



Preparation of (1*R*,2*S*)-2-ethylcyclopropylacetic acid (22) and (1*R*,2*R*)-2-ethylcyclopropylacetic acid (24)

1. Preparation of dioxaborolane ligand 20

1.1 Preparation of Butylmagnesium bromide 17

5 To a 1L, three-necked, round-bottomed flask equipped with a magnetic stirrer, a 125-mL, pressure-equalizing addition funnel, a reflux condenser and a glass stopper, is added 34.0 g (1.40 mol) of magnesium turnings. The agitation is started and the system is flame-dried for 2 min. The flask is then cooled to room temperature under a flow of nitrogen and
10 60 ml of ether is introduced to cover the magnesium. A solution of 48 ml (0.44 mol) of bromobutane in 140 ml of ether is placed in the pressure-equalizing addition funnel. The mixture is heated gently to initiate the reaction. When the reaction has started, the solution of bromide in ether is added dropwise at a rate sufficient to maintain a gentle reflux. After the
15 completion of the addition, the funnel is rinsed with 10 ml of ether. The gray solution is stirred for 20 min and then transferred to a dry flask under nitrogen via cannula. The Grignard reagent is titrated with a solution of isopropanol in benzene using 1,10-phenanthroline as indicator as follows : a dry 10-ml, one necked, round-bottomed flask is charged with 1 ml of
20 Grignard reagent, some drops of dry THF and a crystal of 1,10-phenanthroline. The slightly pink solution is titrated with a solution 0.5 M of isopropanol in dry benzene. Between 3.8 and 4.2 ml are obtained to give a clear colorless solution (3 titrations). A solution of Grignard reagent of 1.90-2.00 M is obtained.

25 **1.2 Preparation of butyl boronic acid 18**

To a 1-L, three-necked, round bottomed flask equipped with a magnetic stirrer and thermometer, was added 480 ml of ether, followed by 20 ml (176 mmol) of trimethylborate. The clear solution was cooled to -75°C (internal

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temperature) and vigorously stirred, then 90 ml (176 mmol) of 1.95 M solution of butylmagnesium bromide in ether was added dropwise *via* cannula at such a rate that the internal temperature did not exceed -65°C . After the addition was completed, the resulting white slurry was stirred for
5 an additional 2 hours at -75°C under nitrogen. The cooling bath was then removed and the reaction mixture was allowed to warm to room temperature (between 1h and 2h are needed). Hydrolysis was carried out by the dropwise addition of 200 ml of a 10% aqueous solution of hydrochloric acid. The white precipitate was dissolved and the resulting clear biphasic mixture was
10 stirred for 15 min, after which time, the two layers were separated. The aqueous layer was extracted with ether (2 x 100 ml), and the combined extracts dried over magnesium sulfate. After concentration of the ethereal solution under reduced pressure, the residual white solid was purified by recrystallization as follows : after dissolution in hot water (50 ml), the
15 resulting biphasic solution was cooled to 0°C to induce recrystallization of the boronic acid. The solid was collected on a medium fritted disk funnel and washed with 100 ml of hexanes and placed under vacuum for 60 min. A quantity of 13.6 g of the boronic acid 18 was produced as a white solid.

Yield : 74.88%

20 m.p. = $94-96^{\circ}\text{C}$ (lit. m.p. = $95-97^{\circ}\text{C}$) (Charette, A. B.; Juteau, H.; Lebel, H.; and Molinaro C. *J. Am. Chem. Soc.* **1998**, *120*, 11943-11952)

Characterization : Nmr (^1H)

1.3 Preparation of [(2-)-N,O,O'[2,2'-iminobis[ethanolato]]]-2-butyl boron 19

25 A 1-L, one-necked, round-bottomed flask equipped with a magnetic stirrer and thermometer, and under nitrogen, was charged with 13,6 g (133 mmol) of the butylboronic acid 18 and 14,0 g (134 mmol) of diethanolamine. Then, 133 ml of dichloromethane and 265 ml of ether were

added, followed by about 27 g of molecular sieve 3Å (powder, dried in an oven overnight at 250°C). The resulting heterogeneous solution was stirred for 1 day under nitrogen. The solid was then triturated with dichloromethane (2 x 100 ml). The filtrate was concentrated under reduced pressure to produce the crude desired complex. The diethanolamine complex was purified by recrystallization as follows : the white solid was dissolved in hot dichloromethane (40 ml), then ether (100 ml) was added to induce crystallization of the complex. The mixture was then cooled to 0°C and the solid was collected on a medium fritted disk funnel and washed with ether (2 x 60 ml). The product was dried under vacuum to afford 18.31 g of the title compound **19** as a white crystalline solid.

Yield : 80%

m.p. = 143-145°C (lit. m.p. = 145-148°C) (Charette, A. B.; Juteau, H.; Lebel, H.; and Molinaro C. *J. Am. Chem. Soc.* **1998**, *120*, 11943-11952).

Characterization : Nmr (¹H)

1.4 Preparation of (R,R)-(+)-N,N,N',N'-tetramethyl tartaric acid diamide **16**

Into a mixture of 68 g (381 mmol) of dimethyl tartrate and 77 ml of methanol spectrograde in a 250 ml Erlenmeyer flask was poured at least 100 ml of liquid, anhydrous, cold (-78°C) dimethylamine (obtained from the condensation of dimethylamine gas at -78°C). The mixture was swirled briefly and allowed to stand in a hood for 3 days with a drying tube in place. After crystallization, the massive crystals were collected by suction filtration. The filtrate was concentrated, seeded, and cooled to yield a second crop. Combined crystals were washed with cold methanol (-30°C) and dried under vacuum. The diamide **16** thus obtained was sufficiently pure to be used in the following step.

Yield : 90 %

m.p. = 188-189°C (lit.189-190°C) (Seebach, D.; Kalinowski, H.-O.; Langer, W.; Wilka, E.-M. *Organic Syntheses*; Wiley : New York, 1990; Coll. Vol. VII, pp 41-50)

Characterization : Nmr (^1H)

5 **1.5 Preparation of (4*R*-trans)-2-butyl-N,N,N',N'-tetramethyl[1,3,2]dioxo-borolane [4,5]dicarboxamide 20**

A 500 mL, one-necked, round-bottomed flask equipped with a magnetic stirrer, and under nitrogen, was charged with 7.00 g (40.9 mmol) of butylboronate diethanolamine 19 complex and 11 g (53.8 mmol) of (*R,R*)-
10 (+)-N,N,N',N'-tetramethyltartaric acid diamide 16. The solids were dissolved upon addition of 205 ml of dichloromethane. Then, 64 ml of brine was added and the resulting biphasic solution was stirred for 2 h 45 min under nitrogen. The two layers were separated and the aqueous layer extracted with dichloromethane (50 ml). The combined organic layers were
15 washed with brine (50 ml), dried over MgSO_4 and filtered. The filtrate was concentrated under reduced pressure and dried vacuum to afford 10,2 g of the title compound 20 as a pale yellow oil.

Yield : 92% (lit. 93%) (Charette, A. B.; Juteau, H.; Lebel, H.; and Molinaro C. *J. Am. Chem. Soc.* 1998, 120, 11943-11952).

20 Characterization : Nmr (^1H , ^{13}C), ir, ms)

2. Preparation of (1*R*,2*S*)-2-ethylcyclopropylacetic acid (22)

2.1 (1*R*,2*S*)-2-ethylcyclopropylethanol 21

A 250-mL, three-necked, round bottomed flask equipped with a magnetic stirrer and a thermometer, and under nitrogen, was charged with 45
25 ml of dry CH_2Cl_2 and 4.6 ml (45 mmol, 4.5 eq.) of dry DME. The solution was cooled to -10°C (internal temperature) with an acetone/ice bath and 4.6 ml (45 mmol, 4.5 eq.) of Et_2Zn was added. To this stirred solution was added 7.2 ml (90 mmol, 9 eq.) of CH_2I_2 over a 1 hour period while

maintaining the internal temperature between -8 and -12°C. After completion of the addition, the resulting solution was stirred for 10 min at -10°C. A solution of 3.36 g (10.8 mmol, 1.2 eq.) of the dioxaborolane 20 ligand in 10 ml of CH₂Cl₂ was added *via* cannula under nitrogen over a 5-6 min period while maintaining the internal temperature below -5°C. A solution of 1.00 g (10 mmol) of *cis*-3-hexen-1-ol in 10 ml of CH₂Cl₂ was immediately added *via* cannula under nitrogen over a 5 - 6 min period while maintaining the internal temperature below -5°C. The cooling bath was removed and the reaction mixture allowed to warm to room temperature and stirred overnight at that temperature.

The reaction was quenched with saturated aqueous NH₄Cl (10 ml) and 10% aqueous HCl (40 ml). The two layers were separated and the aqueous layer was washed with CH₂Cl₂ (50 ml). The combined organic layers were transferred into an Erlenmeyer flask and a solution of 5 M aqueous KOH (200 ml) was added. The resulting biphasic solution was stirred vigorously overnight.

The two layers were then separated and the organic layer was successively washed with saturated aqueous NH₄Cl (3 x 50 ml) and brine (10 ml), dried over MgSO₄, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography (AcOEt/hexanes 2/8) followed by Kugelrohr distillation (150-160°C) to provide the desired cyclopropane derivative 21 (0.9 g).

R_f = 0,33 (AcOEt/hexanes 2/8)

Yield : 79%

[α]_D = + 15.6° (c = 1.66, CHCl₃)

Characterization : Nmr (¹H, ¹³C) , ir, ms

2.2 Preparation of (1*R*,2*S*)-2-ethylcyclopropylacetic acid (22)

A 250-mL, one-necked, round bottomed flask equipped with a magnetic stirrer, and under nitrogen, was charged with 1.65 g (14.3 mmol) of alcohol 21 and 40 ml of dry DMF. Pyridinium dichromate 18.8 g (3.5 eq.;
5 50.1 mmol) was added in one portion. The reaction mixture was stirred overnight at 25°C and then poured into 200 ml of water and extracted with ether (4 x 80 ml). The organic layers were concentrated under reduced pressure, and the residue was then dissolved in chloroform (50 ml) and extracted with a 10% aqueous NaOH (2 x 25 ml). The combined aqueous
10 layers were washed twice with 25 ml of chloroform, followed by acidification with a 10% aqueous HCl. Extraction with ether (4 x 50 ml), drying over MgSO₄, filtration and concentration under reduced pressure afforded the desired compound (1*R*,2*S*)-2-ethylcyclopropylacetic acid (22) as a colorless oil (737 mg).

15 Yield : 39.7%

$[\alpha]_D = + 7.7^\circ$ (c = 3.5, CHCl₃)

Characterization : Nmr (¹H, ¹³C) , ir, ms

3. Preparation of (1*R*,2*R*)-2-ethylcyclopropylacetic acid (24)

3.1 (1*R*,2*R*)-2-ethylcyclopropylethanol 23

20 A 250-mL, three-necked, round bottomed flask equipped with a magnetic stirrer and a thermometer, and under nitrogen, was charged with 45 ml of dry CH₂Cl₂ and 4.67 ml (45 mmol, 4.5 eq.) of dry DME. The solution was cooled to -10°C(internal temperature) with an acetone/ice bath and 4.6 ml (45 mmol, 4.5 eq.) of Et₂Zn were added. To this stirred solution was
25 added 7.2 ml (90 mmol, 9 eq.) of CH₂I₂ over a 1 hour period while maintaining the internal temperature between -8 and -12°C. After completion of the addition, the resulting solution was stirred for 10 min at -10°C. A solution of 3.36 g (10.8 mmol, 1.2 eq.) of the dioxaborolane 20

ligand in 10 ml of CH_2Cl_2 was added *via* cannula under nitrogen over a 5–6 min period while maintaining the internal temperature below -5°C . A solution of 1.00 g (10 mmol) of *trans*-3-hexen-1-ol in 10 ml of CH_2Cl_2 was immediately added *via* cannula under nitrogen over a 5 – 6 min period while
5 maintaining the internal temperature below -5°C . The cooling bath was removed and the reaction mixture allowed to warm to room temperature and stirred overnight at that temperature.

The reaction was quenched with saturated aqueous NH_4Cl (10 ml) and 10% aqueous HCl (40 ml). The two layers were separated and the
10 aqueous layer was washed with CH_2Cl_2 (50 ml). The combined organic layers were transferred into an Erlenmeyer flask and a solution of 5 M aqueous KOH (200 ml) was added. The resulting biphasic solution was stirred vigorously overnight.

The two layers were then separated and the organic layer was
15 successively washed with saturated aqueous NH_4Cl (3 x 50 ml) and brine (10 ml), dried over MgSO_4 , filtered and concentrated under reduced pressure. The residue was purified by flash chromatography (AcOEt /hexanes 2/8) followed by Kugelrohr distillation ($150\text{--}162^\circ\text{C}$) to provide the desired cyclopropane derivative **23** (1.06 g).

20 $R_f = 0.33$ (AcOEt /hexanes 2/8)

Yield : 93%

$[\alpha]_D = + 6.22^\circ$ ($c = 2.25$, CHCl_3) (lit. $[\alpha]_D = + 9.6^\circ$ ($c = 0.3$, CHCl_3) (Charette, A. B.; Juteau, H.; Lebel, H.; and Molinaro C. *J. Am. Chem. Soc.* 1998, 120, 11943-11952).

25 Characterization : Nmr (^1H , ^{13}C), ir, ms

3.2 Preparation of (1*R*,2*R*)-2-ethylcyclopropylacetic acid (**24**)

A 250-mL, one-necked, round bottomed flask equipped with a magnetic stirrer, and under nitrogen, was charged with 1.7 g (14.9 mmol) of

alcohol 23 and 40 ml of dry DMF. Pyridinium dichromate 19.6g (3.5 eq.; 52.1 mmol) was added in one portion. The reaction mixture was stirred overnight at 25°C and then poured into 200 ml of water and extracted with ether (4 x 80 ml). The organic layers were concentrated under reduced pressure, and the residue was then dissolved in chloroform (50 ml) and extracted with a 10% aqueous NaOH (2 x 25 ml). The combined aqueous layers were washed twice with 25 ml of chloroform, followed by acidification with 10% aqueous HCl. Extraction with ether (4 x 50 ml), drying over MgSO₄, filtration and concentration under reduced pressure afforded the desired compound (1*R*,2*R*)-2-ethylcyclopropylacetic acid (24) as a colorless oil (1.05 g).

Yield : 55.5%

$[\alpha]_D = + 4.36^\circ$ (c = 1.83, CHCl₃)

Characterization : Nmr (¹H, ¹³C) , ir, ms

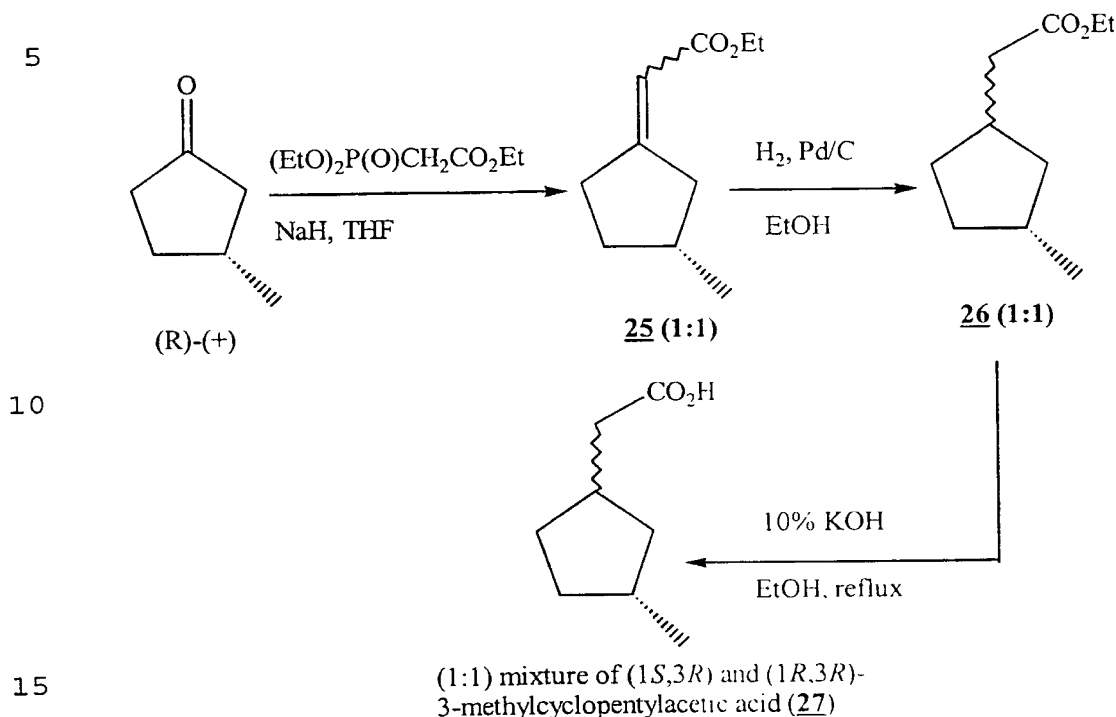
EXAMPLE VII

Synthesis of a (1:1) mixture of (1*S*,3*R*) and (1*R*,3*R*)- 3-methylcyclopentylacetic acid (27)

The (1:1) mixture of (1*S*,3*R*) and (1*R*,3*R*)- 3-methylcyclopentylacetic acid (27) was synthesized according to the outline in scheme 5. A Wittig-Horner (Duraismy, M. and Walborsky H. M. *J. Am. Chem. Soc* **1983**, *105*, 3252-3264) reaction involving condensation of (*R*)(+)-3-methyl cyclopentanone and triethyl phosphonoacetate gave a (1 : 1) mixture of ethyl (*E*, and *Z*,3*R*)-(3-methyl cyclopentylidene) carboxylate 25. Hydrogenation of the α,β-unsaturated ester 25 proceeded without stereocontrol, and a (1 : 1) mixture of the ester 26 was obtained in quantitative yield. Hydrolysis of the ester 26 with alcoholic KOH afforded a

(1 : 1) mixture of the desired (1*S*,3*R*) and (1*R*,3*R*)- 3-methylcyclopentylacetic acid (27) in 92% yield.

Scheme 5



Preparation of a (1:1) mixture of (1*S*,3*R*) and (1*R*,3*R*)- 3-methylcyclopentylacetic acid (27)

1. Preparation of α,β -unsaturated ester 25

To a dry 100 ml, one necked, round-bottomed flask equipped with a magnetic stirrer, was added 611 mg (15.8 mmol) of 60% sodium hydride as a dispersion in mineral oil. The oil was removed by washing twice with pentane. Dry THF (21 ml) was added and the suspension, maintained under nitrogen atmosphere. After cooling the flask to 0°C in an ice bath, 3.43 g (15.2 mmol) of triethyl phosphonoacetate was slowly added. Stirring was continued for 35 min, and *R*-(+)-3-methylcyclopentanone (1.51 g; 15.2 mmol) was then added, and the temperature was kept at room temperature for an additional hour, during which time a gelatinous precipitate appeared.

Ether (100 ml) and water (50 ml) were added to quench the reaction. The mixture was transferred to a separatory funnel and washed twice with water and then with brine. The organic solution was dried over MgSO_4 , filtered and concentrated under reduced pressure. The residue was purified by flash chromatography (hexane/ AcOEt , 9/1) to afford 2.4 g (96%) of the desired product 25 as a clear, colorless oil.

$R_f = 0.53$ (hexane/ AcOEt , 9/1)

Characterization : Nmr (^1H , ^{13}C), ir, ms

2. Preparation of ester 26

To a stirred solution of α,β -unsaturated ester 25 (1.3 g; 7.7 mmol) in anhydrous ethanol (68 ml) was added 163 mg of 10% palladium on carbon. The mixture was stirred overnight under an atmosphere of hydrogen. The catalyst was then removed by filtration on a pad of celite. The filtrate was concentrated and the residue, ester 26, a colorless oil, was used without purification for the next step.

Yield : (1.2 g) 92 %.

Characterization : Nmr (^1H , ^{13}C), ir, ms

3. Preparation of a (1:1) mixture of (1*S*,3*R*)- and (1*R*,3*R*)- 3-methyl cyclopentylacetic acid (27)

To the ester 26 (1.20 g; 7.05 mmol) in ethanol (50 ml) was added 15 ml of a 10% aqueous solution of potassium hydroxide. The reaction mixture was stirred under reflux for 5 hours. The ethanol was then evaporated in vacuo and the residue extracted with ether (20 ml). The aqueous phase was acidified with 10% aqueous HCl and extracted with ether (3 x 50 ml). The combined organic phases were then dried over MgSO_4 , filtered and concentrated under reduced pressure to give a (1:1) mixture of (1*S*,3*R*)- and (1*R*,3*R*)- 3-methyl cyclopentylacetic acid (27) as a colorless oil (920 mg) in 92% yield.

Characterization : Nmr (^1H , ^{13}C) , ir, ms

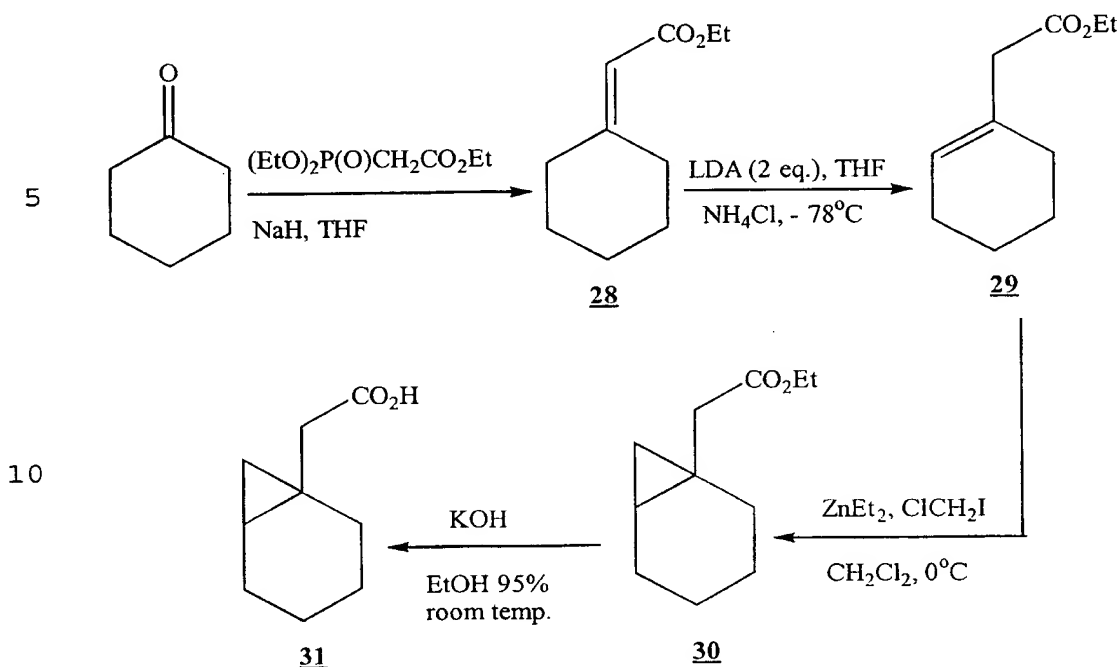
EXAMPLE VIII

Synthesis of bicyclo[4.1.0]heptylacetic acid (31)

- 5 The compound 31 was prepared in four steps as outlined in scheme 6. Application of the Wittig-Horner procedure (Duraismy, M. and Walborsky H. M. *J. Am. Chem. Soc* **1983**, *105*, 3252-3264) to cyclohexanone with triethyl phosphonoacetate (NaH, THF) provided the α,β -unsaturated ester 28 in 88% yield. The desired β,γ -unsaturated ester 29
- 10 could be prepared from the α,β -unsaturated ester 28 by kinetic trapping of the extended enolate (LDA, THF) with saturated aqueous NH_4Cl at -78°C . Cyclopropanation (Scott E. Denmark and James P. Edwards *J. Org. Chem.* **1991**, *56*, 6974-6981) with diethylzinc/chloriodomethane in dichloromethane at 0°C produced the cyclopropyl 30 in 91.5% yield.
- 15 Saponification of ester 30 in KOH/EtOH followed by subsequent acidification afforded 31 in 92% yield (Kantorowski, E. J.; Eisenberg, S. W. E.; Fink, W. H.; and Kurth, M. J. *J. Org. Chem.*, **1999**, *64*, 570-580).

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PCT/CA99/00816

Scheme 6



Preparation of bicyclo[4.1.0]heptylacetic acid (31)

15 1. Preparation of α,β -unsaturated ester 28

To a dry 200 ml, one necked, round-bottomed flask equipped with a magnetic stirrer, was added 1.22 g (30.5 mmol) of 60% sodium hydride as a dispersion in mineral oil. The oil was removed by washing twice with pentane. Dry THF (50 ml) was added and the suspension, maintained under nitrogen atmosphere. After cooling the flask to 0°C in an ice bath, 6.85 g (30.5 mmol) of triethyl phosphonoacetate was slowly added. Stirring was continued for 30 min, and cyclohexanone (3.00 g; 30.5 mmol) was then added, and the reaction mixture was kept at room temperature for an additional hour, during which time a gelatinous precipitate appeared. Ether (50 ml) and water (50 ml) were added to quench the reaction. The mixture was transferred to a separatory funnel and washed twice with water and then with brine. The organic solution was dried over MgSO_4 , filtered and concentrated under reduced pressure. The residue was purified by flash

chromatography (hexane/AcOEt, 9.5/0.5) to afford 4.52 g (88%) of the desired product 28 as clear, colorless oil.

Rf = 0.56 (hexane/AcOEt, 9.5/0.5)

Characterization : Nmr (^1H , ^{13}C) , ir, ms

5 **2. Preparation of β,γ -unsaturated ester 29**

To a solution of diisopropylamine (3.33 ml; 23.7 mmol) in dry THF (90 ml), kept at 0°C, was slowly added n-butyllithium (11.9 ml; 23.7 mmol). The solution was stirred for 20 min and then cooled to -78°C. The α,β -unsaturated ester 28 (2.00 g, 11.9 mmol) in THF (9 ml) was added dropwise over 10 min and the mixture was allowed to stir for 30 min at that temperature. Saturated aqueous NH_4Cl (20 ml) was added dropwise over a 10 min period, and the quenched reaction was allowed to warm to ambient temperature before being poured in water (10 ml) and extracted with ether (3 x 50 ml). The extracts were dried (MgSO_4), filtered and concentrated to afford a mixture of (4 : 1) β,γ -unsaturated : α,β -unsaturated ester (nmr) as a yellow oil. Flash chromatography (hexane/AcOEt, 9.65/0.35) afforded 1 g (50 %) of the desired product 29 as clear, colorless oil.

Rf = 0.44 (hexane/AcOEt, 9.5/0.5)

Characterization : Nmr (^1H , ^{13}C) , ir, ms

20 **3. Preparation of cyclopropyl ester 30**

In a 100 ml, one necked flask, a solution of Et_2Zn (1.27 ml; 11.0 mmol; 2 eq.) in dry dichloromethane (20 ml) was cooled to 0°C, and a solution of ClCH_2I (1.6 ml; 22 mmol; 4 eq.) was added *via* syringe. The solution was stirred for 5 min at 0°C, during which time a precipitate was formed, and a solution of β,γ -unsaturated ester 29 (924 mg, 5.49 mmol) in CH_2Cl_2 (8 ml) was added *via* cannula. The reaction mixture was stirred for 60 min at 0°C and quenched with a saturated aqueous solution of NH_4Cl (60ml). The solution was then allowed to warm to room temperature, stirred

vigorously for 10 min, and extracted with ether (3 x 20 ml). The extracts were washed with H₂O (1 x 20 ml) and brine (1 x 20 ml), combined, dried (MgSO₄), and concentrated under reduced pressure. The residue was purified by flash chromatography (hexane/AcOEt, 9.5/0.5) to afford 915 mg (91.5%) of the desired product **30** as clear, colorless liquid.

R_f = 0.44 (hexane/AcOEt, 9.5/0.5)

Characterization : Nmr (¹H, ¹³C) , ir, ms

4. Preparation of bicyclo[4.1.0]heptylacetic acid (**31**)

Ester **30** (600 mg; 3.29 mmol) in basic ethanol (2.09 g KOH in 27 ml 95% ethanol) was stirred at room temperature overnight. The reaction was diluted with ether and extracted with NaOH (2 M, 2 x 50 ml), the combined extracts were acidified with aqueous 10% HCl and extracted with ether (3 x 40 ml). The combined organics were dried (MgSO₄), filtered and concentrated to give the desired **31** as a pale yellow liquid (467 mg; 92%).

Characterization : Nmr (¹H, ¹³C) , ir, ms

EXAMPLE IX

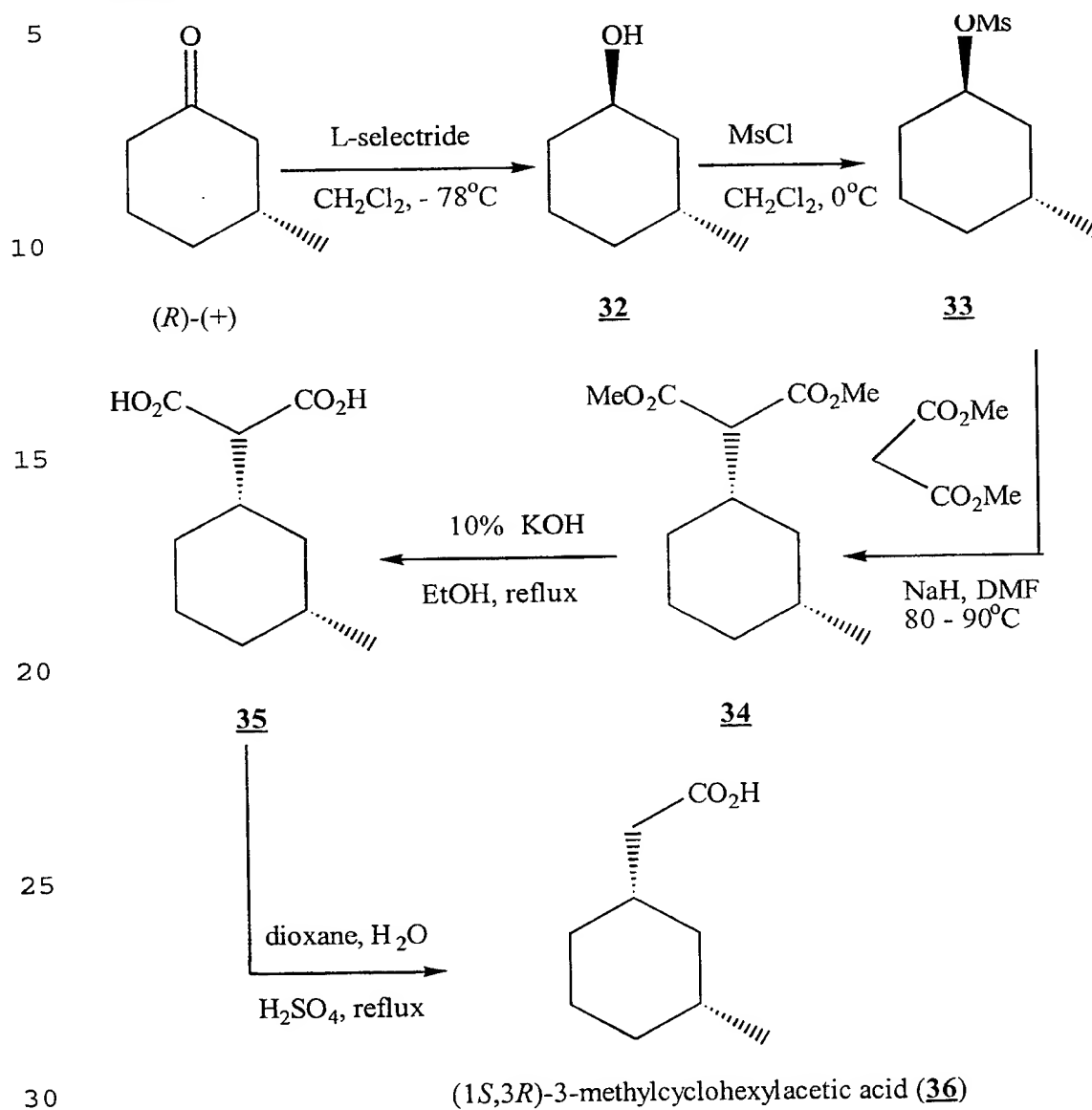
Synthesis of (1*S*, 3*R*)-3-methylcyclohexylacetic acid (**36**)

Finally, the synthesis of (1*S*, 3*R*)-3-methylcyclohexylacetic acid (**36**) was accomplished as depicted in scheme 7. First, (*R*)-(+)-3-methylcyclohexanone was reduced in 75% yield according to Brown's method (Brown, H. C.; Jadhav, P. K. In ``Asymmetric Synthesis`` Morisson, J. D., Ed.; Academic Press : New York, 1983; Vol. II, Chapter 1; Brown, H. C.; Desai, M.C.; Jadhav, P. K. *J. Org. Chem.* 1982, 47, 5065-5069) to (3*R*,1*R*)-3-methylcyclohexanol **32**.

The optically active **32** was mesylated and reacted with the sodium salt of dimethyl malonate in dry DMF at 80 – 90°C to produce the desired diester **34** in 31% yield. Hydrolysis of the diester **34** with alcoholic KOH afforded the diacid **35** which upon decarboxylation under rather

drastic conditions (concentrated H_2SO_4 in dioxane/water mixture under reflux for two days) gave the desired compound (1*S*, 3*R*)-3-methylcyclohexylacetic acid (**36**) in 74.8% yield.

Scheme 7



Preparation of (1*S*, 3*R*)-3-methylcyclohexylacetic acid (36)

1. Preparation of alcohol 32

A stirred solution of *R*-(+)-3-methylcyclohexanone (3.50 g; 31.1 mmol) in dry THF (40 ml) at -78°C was treated with a 1.0 M solution of L-selectride in THF (63 ml; 63 mmol). After 2 hours of stirring at that
5 temperature, a solution of 2 M aqueous sodium hydroxide (33 ml) was added followed by 23 ml of 30% H₂O₂. After warming to room temperature the reaction mixture was treated with 10% aqueous HCl and the product was isolated by extraction with ether (3 x 80 ml). The extracts were combined,
10 dried over (MgSO₄), filtered and concentrated under vacuo. The residue was purified by flash chromatography (hexane/AcOEt, 8/2) to afford 2.70 g (75%) of the desired product 32 as a clear, colorless oil.

R_f = 0.52 (hexane/AcOEt, 8/2)

[α]_D = -2.90 (c = 7.9; chloroform)

15 Characterization : Nmr (¹H, ¹³C) , ir, ms

2. Preparation of mesylate 33

To a stirred solution of (1*R*,3*R*)-(-)-3-methyl cyclohexanol (32) (1.27 g; 11.1 mmol) in dry dichloromethane (45 ml) were added Et₃N (2.3 eq.; 25.7 mmol; 3.58 ml) and methanesulfonyl chloride (1.2 eq.; 13.4 mmol;
20 1.00 ml) in a dropwise fashion at 0°C. The reaction mixture was stirred for 1 hour at 0°C, and then diluted with dichloromethane (80 ml) and 50 ml of 10% aqueous HCl. The aqueous phase was extracted with CH₂Cl₂ (50 ml). The combined organic layers were successively washed with saturated NaHCO₃ (1 x 20 ml) and brine (1 x 20 ml) and then dried over MgSO₄,
25 filtered and concentrated in vacuo to afford (2.13 g) of crude mesylate 33 as a yellow oil which was used without purification for the next step.

R_f = 0.52 (hexane/AcOEt, 8/2)

Characterization : Nmr (¹H, ¹³C)

3. Preparation of diester 34

To a dry 200 ml, one necked, round-bottomed flask equipped with a magnetic stirrer, was added (3.5 eq.; 39 mmol; 1.16 g) of 60% sodium hydride as a dispersion in mineral oil. The oil was removed by washing
5 twice with pentane. Dry DMF (30 ml) was added and the suspension, maintained under nitrogen atmosphere. After cooling the flask to 0°C in an ice bath, dimethyl malonate (3.5 eq.; 39 mmol; 5.16 g) was added. Stirring was continued for 10 min, and a solution of the mesylate 33 in DMF (58 ml)
10 was added *via* cannula. The mixture was then stirred and heated at 80 – 90°C for 3 days. Water (50 ml) was then added and the mixture was transferred to a separatory funnel and extracted with ether (3 x 100 ml). The organics were dried over MgSO₄, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography (hexane/AcOEt, 8/2) to afford
15 800 mg (31%) of the desired product 34 as clear, colorless oil.

R_f = 0.49 (hexane/AcOEt, 8/2)

Characterization : Nmr ¹H

4. Preparation of diacid 35

To a solution of the diester 34 (800 mg; 3.50 mmol) in 53 ml of
20 ethanol, was added 8 ml of a 10% aqueous solution of potassium hydroxide. The mixture was heated to reflux for 5 hours, then left under stirring overnight. Ethanol was evaporated under reduced pressure and the residue was diluted with water (10 ml) and extracted with ether (20 ml). The aqueous phase was acidified with 10% aqueous HCl, then extracted with
25 ether (3 x 30 ml). After drying over MgSO₄, filtration and concentration under *vacuo* we obtained a white crystalline solid which was used without purification for the next step.

Yield : (600 mg) 85.6 %

Characterization : Nmr ^1H (CD_3OD)

5. Preparation of (1*S*, 3*R*)-3-methylcyclohexylacetic acid (36)

To the diacid 35 (800 mg, 4.00 mmol) was added 15 ml of dioxane, 5 ml of water and 2 ml of sulfuric acid. The mixture was then heated to reflux for 3 days. After cooling to room temperature and extraction with ether (3 x 30 ml), the combined organic phases were dried over MgSO_4 , filtered and concentrated to give (1*S*, 3*R*)-3-methylcyclohexylacetic acid (36) as a yellow oil.

Yield : (467 mg) 74.8%

Characterization : Nmr (^1H , ^{13}C) , ir, ms

EXAMPLE X

Formation of hydrophobic GRF analogs

The coupling of X moiety to GRF-peptide to result in the formation of the hydrophobic GRF analog of the present invention was chemically synthesized by anchoring one or several hydrophobic tails at the N- terminal portion of GRF or one of its analogs as described above.

More precisely, for a better carrying out of the chemical anchoring reaction, hydrophobic functionalized under the acid form are preferably used. In these conditions, the anchoring reaction is preferably effected in a solid phase (Merrifield R.B., 1963, *J. Am. Chem. Soc.*, **85**:2149; 1964, *J. Am. Chem. Soc.*, **86**:304) using extremely active reagents such as for example Benzotriazol-1-yloxytris (dimethylamino) phosphonium hexafluorophosphate known in the prior art (B. Castro et al., 1975, *Tetrahedron letters*, Vol. **14**:1219).

In the case where the hydrophobic tail to be anchored consists in a fatty acid, the activation in view of the anchoring may be carried out *in situ*. Depending on the synthesis strategies used, the peptide anchoring site is liberated just prior to the anchoring in traditional deprotection conditions

(Gross et Meienhofer, 1981, *The peptides*, vol. 3, Academic press: pages 1-341). The hydrophobic tail (Ht) is then condensed with the anchoring agent in organic solvents such as an ether (tetrahydrofuran), an aliphatic halogenated solvent (dichloromethane), a nitrile (acetonitrile) or an amide
5 (N,N-dimethylformamide).

With respect to the anchoring dynamic, the preferred working temperatures are between 20 and 60°C. The anchoring reaction time when hydrophobic tail used are more and more hydrophobic, varies inversely with temperature, but varies between 0.1 and 24 hours.

10 General GRF analogs synthesis steps were carried out by solid-phase methodology on a 9050TM plus peptide synthesizer (Millipore Corporation, Milford, MA) using Fmoc strategy and synthesis cycles supplied by Millipore. Fmoc amino acids were supplied by Bachem California and other commercial sources. Sequential Fmoc chemistry using
15 BOP/HOBt as coupling methodology were applied to the starting Fmoc-Pal-PEG resin (Millipore, catalog number: GEN 913383) for the production of C-terminal carboxamides. Fmoc deprotections were accomplished with piperidine 20% solution in DMF. After synthesis completion, the resin was well washed with DMF and ether prior to drying. Final cleavages of side
20 chain protecting groups and peptide-resin bonds were performed using Millipore supplied procedure consisting of the following mixture: TFA, water, phenol, triisopropylsilane (88:5:5:2). Peptides were then precipitated and washed with ether prior to drying. Reverse phase HPLC purification (buffer A: TEAP 2.5; buffer B: 80% CH₃CN in A) using a waters prep
25 4000, absorbance 214nm, detector model 486, flow rate 50ml/min.; linear gradient generally from 25 to 60%B in 105 min.) followed by a desalting step (buffer C:0.1% TFA in H₂O; buffer D:0.1% TFA in CH₃CH/H₂O 80:20) afforded peptides in yields amounting from 10 to 30% with

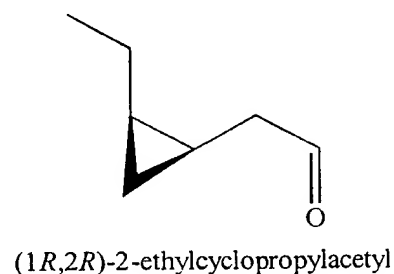
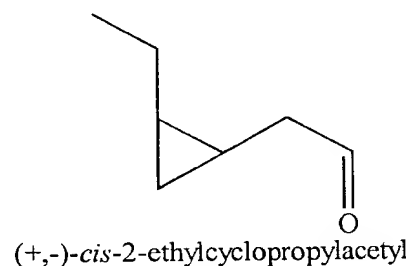
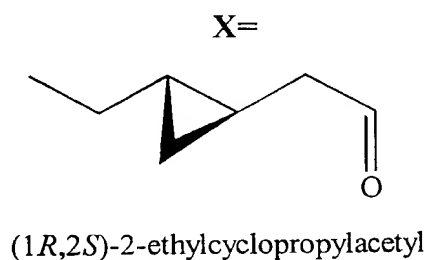
homogeneity greater than 97% as estimated by HPLC (millennium/photodiode array detection).

The above procedure was used to synthesize seven new GRF analogs of formula A:

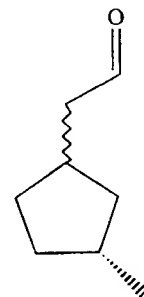


wherein X is the acyl portion of the corresponding carboxylic acids X-OH synthesized in examples VI, VII, VIII and IX.

analog

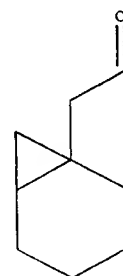


4



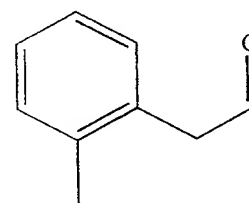
(1:1) mixture of (1*S*,3*R*) and (1*R*,3*R*)-
3-methylcyclopentylacetyl

5



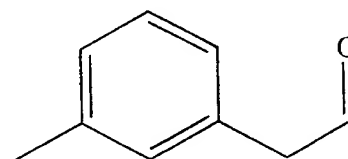
bicyclo [4.1.0]heptylacetyl

6



2-methylphenylacetyl

7



3-methylphenylacetyl

EXAMPLE XI

Effect of 8 different hGRF(1-44)NH₂ analogs on IGF-1 levels

The objective of this experiment was to compare the effects of 8 different hGRF(1-44)NH₂ analogues including TH 9507 on IGF-1 levels following S.C. chronic administration in growing male pigs.

INS

B1

5

Animal procedures were conducted as described in the protocol GRF-30 submitted April 30, 1999, with the following modification: only 8 GRF analogues, including TH 9507, were provided by the sponsor. Accordingly, the study was performed on 30 pigs.

5 Laboratory procedures were conducted as follow: IGF-1 levels were measured in pig sera using a commercial kit (DSL-5600) purchased from Diagnostic Systems Laboratories Inc., Tx, USA. Briefly, IGF-1 levels are quantified using a two-site immunoradiometric assay (IRMA) following acid-ethanol extraction.

10 Statistical analysis: IGF-1 data were subjected to a two-way repeated measures analysis of variance, with time (day 1, 3 or 6) and treatments (A to J) as variation factors pairwise multiple comparison procedures were then run by the Student-Newman-Keuls method. All statistical procedures were performed using the computerized SigmaStat
15 software (Jandel Scientific). A $P < 0.05$ was considered statistically significant.

As shown in Fig. 7, neither saline nor hGRF(1-44)NH₂-treated animals had any significant variations of their IGF-1 concentrations in the study period. This confirm previous results showing that hGRF(1-44)NH₂ is
20 not able to induce any significant increase in IGF-1 levels when injected subcutaneously in pigs twice a day at 10 ug/kg.

All tested analogs (TH 9507 and analogues 1 to 7) significantly increased IGF-1 levels in pigs on day 6, when compared to their basal levels on day 1. Only analogues 3 and 6 significantly increased IGF-1 levels on
25 day 3. No significant difference between analogues was derived from the statistical analysis. However, analogue 6 appeared to exhibit a very strong IGF-1 inducing efficacy, bringing IGF-1 levels up to 358.3 ± 17.1 ng/ml following 5 days of injection (when compared to 190.1 ± 21.0 ng/ml and

279.1 ± 28.4 ng/ml in the saline- and the TH 9507-treated groups, respectively.

These results indicate that all 7 new hGRF(1-44)NH₂ analogs tested in the present invention are more potent than the native GRF molecule. This suggests that the attachment of various aliphatic chains on the N-terminal portion of GRF(1-44)NH₂ successfully increases its GH-releasing potency.

1990-1991		1991-1992		1992-1993		1993-1994		1994-1995		1995-1996		1996-1997		1997-1998		1998-1999		1999-2000		2000-2001		2001-2002		2002-2003		2003-2004		2004-2005		2005-2006		2006-2007		2007-2008		2008-2009		2009-2010		2010-2011		2011-2012		2012-2013		2013-2014		2014-2015		2015-2016		2016-2017		2017-2018		2018-2019		2019-2020		2020-2021		2021-2022		2022-2023		2023-2024		2024-2025		2025-2026		2026-2027		2027-2028		2028-2029		2029-2030		2030-2031		2031-2032		2032-2033		2033-2034		2034-2035		2035-2036		2036-2037		2037-2038		2038-2039		2039-2040		2040-2041		2041-2042		2042-2043		2043-2044		2044-2045		2045-2046		2046-2047		2047-2048		2048-2049		2049-2050		2050-2051		2051-2052		2052-2053		2053-2054		2054-2055		2055-2056		2056-2057		2057-2058		2058-2059		2059-2060		2060-2061		2061-2062		2062-2063		2063-2064		2064-2065		2065-2066		2066-2067		2067-2068		2068-2069		2069-2070		2070-2071		2071-2072		2072-2073		2073-2074		2074-2075		2075-2076		2076-2077		2077-2078		2078-2079		2079-2080		2080-2081		2081-2082		2082-2083		2083-2084		2084-2085		2085-2086		2086-2087		2087-2088		2088-2089		2089-2090		2090-2091		2091-2092		2092-2093		2093-2094		2094-2095		2095-2096		2096-2097		2097-2098		2098-2099		2099-2100		2100-2101		2101-2102		2102-2103		2103-2104		2104-2105		2105-2106		2106-2107		2107-2108		2108-2109		2109-2110		2110-2111		2111-2112		2112-2113		2113-2114		2114-2115		2115-2116		2116-2117		2117-2118		2118-2119		2119-2120		2120-2121		2121-2122		2122-2123		2123-2124		2124-2125		2125-2126		2126-2127		2127-2128		2128-2129		2129-2130		2130-2131		2131-2132		2132-2133		2133-2134		2134-2135		2135-2136		2136-2137		2137-2138		2138-2139		2139-2140		2140-2141		2141-2142		2142-2143		2143-2144		2144-2145		2145-2146		2146-2147		2147-2148		2148-2149		2149-2150		2150-2151		2151-2152		2152-2153		2153-2154		2154-2155		2155-2156		2156-2157		2157-2158		2158-2159		2159-2160		2160-2161		2161-2162		2162-2163		2163-2164		2164-2165		2165-2166		2166-2167		2167-2168		2168-2169		2169-2170		2170-2171		2171-2172		2172-2173		2173-2174		2174-2175		2175-2176		2176-2177		2177-2178		2178-2179		2179-2180		2180-2181		2181-2182		2182-2183		2183-2184		2184-2185		2185-2186		2186-2187		2187-2188		2188-2189		2189-2190		2190-2191		2191-2192		2192-2193		2193-2194		2194-2195		2195-2196		2196-2197		2197-2198		2198-2199		2199-2200		2200-2201		2201-2202		2202-2203		2203-2204		2204-2205		2205-2206		2206-2207		2207-2208		2208-2209		2209-2210		2210-2211		2211-2212		2212-2213		2213-2214		2214-2215		2215-2216		2216-2217	
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